The role of APC (Anaphase-Promoting Comlpex) in G2/M after DNA damage

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Le rôle du APC (Anaphase-Promoting Complex) au cours de la phase G2/M après dommage de l'ADN

The role of APC (Anaphase-Promoting Comlpex) in G2/M after DNA damage

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RESUME

Les agents permettant de créer des dommages sur l'ADN sont principalement utilisés dans les traitements contre le cancer. L'activation de points de contrôle du cycle cellulaire après lésion de l'ADN entraîne un arrêt du cycle des cellules. De la connaissance des mécanismes moléculaires de l'arrêt du cycle cellulaire par ces points de contrôle dépend l'efficacité du traitement. Dans les cellules humaines, ces points de contrôle sont primordiaux puisque leur inactivation entraîne la carcinogenèse (génération de cancers). Après traitement par des agents chimiothérapiques et des rayons X, les cellules s'arrêtent en phase G-1 et G-2/Mitose (M) du cycle cellulaire. Si de nombreuses études ont permis de clarifier les mécanismes de l'arrêt en phase G-1 pour des cellules dont l'ADN est endommagé, peu de données sont disponibles concernant l'arrêt en phase G-2/M. Parmi ces points de contrôle, le point de contrôle G-2/M est particulièrement important car il prévient l'entrée en mitose (phase M) des cellules dont l'ADN est endommagé.

Nous avons analysé le rôle du complex appelé APC (Anaphase-Promoting Complex) dans les points de contrôle G-2/M après lésion de l'ADN. Les lésions de l'ADN sont induites dans les cellules synchronisées en phase S. Suite à ces dommages, les cellules montrent un retard et s'arrêtent en phase G-2 avec 4N chromosomes. Afin d'identifier les bases biochimiques de l'arrêt en G-2/M après traitement avec des agents endommageant l'ADN, nous allons concentrer notre recherche sur un complexe composé de multiples protéines possédant une activité de ligation de l'ubiquitine de type E3 (ubiquitin-ligase E3). Ce complexe APC est necessaire pour la dégradation des inhibiteurs d'entrée en anaphase, cyclins mitotiques, et plusieurs kinases mitotiques pour la complétion de la sortie de la mitose. Nous avons analysé et déterminé que l'absence d'activité du complexe APC inhibe l'activation du point de contrôle G-2/M lors de dommages de l'ADN.

Mots clés

Cycle cellulaire – Points de contrôle – APC (Anaphase Promoting Complex) - Aneuploïdie – Mitose - pRb – p21 – Cycline - CDK - Emi1 – Replication de l'ADN – Roscovitine - Fuseau mitotique – Nocodazole – Cancer

ABSTRACT

DNA damaging agents are the most widely used treatment in fight against cancer. The effective use of DNA damaging agents for killing tumors depends on understanding the mechanism of DNA damage checkpoint arrest at the molecular level. DNA damage checkpoints impose delays in cell cycle in response to DNA damage. Cells arrest in G2/M after treatment with DNA-damaging agents, such as chemotherapeutic agents and x-rays. In human cells DNA damage checkpoints are of critical importance in carcinogenesis since inactivation of the checkpoint leads to increased rates of mutation, chromosomal loss or aneuploidy. While G-1 arrest after DNA damage has been extensively studied, the mechanism of G2 arrest is less clear. Among the cell cycle checkpoints, G2 is most crucial for preventing entry into mitosis with damaged DNA.

We have found a previously unrecognized link between anaphase promoting complex (APC) and G2 checkpoint control after DNA damage. The APC is a large multi-protein complex with E3-ubiquitin ligase activity. APC is best known for regulating progression through mitosis and mitotic exit activity by degradation of various mitotic substrates. APC activity is high from late mitosis until late G-1 phase of the cell cycle. We surprisingly find that APC is activated following DNA damage in cells arrested in G2. DNA damage was induced in synchronized cells in late S phase. Following DNA damage, cells show G2 delay and remain arrested in with a DNA content of 4N. Importantly, we show that down-regulation of APC activity by siRNA technique abolishes G2 checkpoint control after DNA damage. We've analyzed how DNA damage that leads to APC activation. The specific destruction of a regulator by APC may govern cell cycle arrest after DNA damage.

Key words

Cell cycle – Checkpoint – APC (Anaphase Promoting Complex) - Aneuploidy – Mitosis - pRb – p21 – Cyclin - CDK - Emi1 – DNA replication – Roscovitine - mitotic spindle – Nocodazole – Cancer

TABLE DES MATIERES

ABBREVIATIONS

APC/C : anaphase promoting complex / Cyclosome
ATM : ataxia telangiectsia mutated
ATR : ataxia telangiectsia and Rad3-related
ATRIP : ATR interacting protein
BRG1 : brm/SW12-related gene 1
β-TrCP : beta-transducin repeat containing protein
BrdU : bromodeoxyuridine triphosphate
BSA : bovine albumine serum
BUB : budding uninhibited by benomyl
CAK : CDK-activating kinase
CDC : cyclin division control
CDK : cyclin dependent kinase
CIN : chromosome instability
Claspin : Chk1 large associated protein
CKI : CDK inhibitor
CtIP : CtBP-interacting protein
DABCO : 1-4-diazabicyclo (2,2,2) octane
DMSO : dimethylsulfoxide
DNA : deoxyribonucleic acid
ECL : Electrochemiluminescence
EDTA : ethylenediamine tetraacetate
Emi1/2 : early mitotic inhibitor 1/2
FBP : F-box protein
FEN1 : Flap endonuclease 1
FITC : fluorescein isothiocyanate
HDAC : histone deacetylase
HEPES : *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HRP : horseradish peroxidase
HU : hydroxyurea
IP : Immunoprecipitations
LLR : Leucine-rich repeat
MAD : mitotic arrest deficient
MAPK : Mitogen-activated protein kinase
MCM : minichromosome maintenance protein
MEF : mouse embryonic fibroblast
MPF : M-phase promoting factor
MPS1 : monopolar spindle 1
MTI : microtubule inhibitor
NP-40 : nonidet-40
ORC : origin recognition complex
PAGE : polyacrylamide gel electrophoresis
PAS or PGS : Protein A(or G)-Sepharose
PBS : phosphate-buffered saline

PCNA : Proliferating Cell Nuclear Antigen
Plk1 : polo-like kinase 1
PMSF : phenylmethylsulfonide fluoride
Rb : retinoblastoma protein
RFC : replication factor C
RNA : ribonucleic acid
RPA : replication protein A
Pre-RC : prereplicative complex
SCF : Skp1 / Culin / F-box protein
SDS : sodium dodecyl sulfate
Skp2 : S-phase kinase associated protein 2
Tris : tris(hydroxymethyl) aminomethane
UBC : ubiquitin-congugating enzyme
UV : ultraviolet
ZBR : zinc binding region

ILLUSTRATIONS

TBLEAUX

INTRODUCTION

Première Partie : La Cycle Cellulaire << Cell Cycle >>

In this section, I will describe the « somatic » eukaryotic cell cycle which permits one cell to obtain two identical daughter cells. I will not address other cell cycle types here. For example, meiosis has specific chromosome reduction phase which shows from diploid cell to haploid cell (germinal cell), or multinucleic cells by replication and separation of chromosomes without cellular cleavage (syncytiums).

A. Cell Cycle

The main purpose of cell cycle in most case is to produce two daughter cells with accurate copies of the parent. The cell cycle is divided into four major phases. In somatic cells, chromosomes are duplicated during the S (synthesis) phase. After completing DNA replication, cells progress to G2 (Gap) phase and begin to prepare their division in M (mitosis) phase, which is divided into several stages. Chromosome condensation occurs during prophase and nuclear envelope is disrupted in prometaphase. Sister chromatids attach to the microtubule and align along their length in the center of cell equidistantly between two poles of the mitotic spindle during metaphase. During anaphase, each sister chromatid migrates to opposite poles of spindle and separates to each daughter cell.

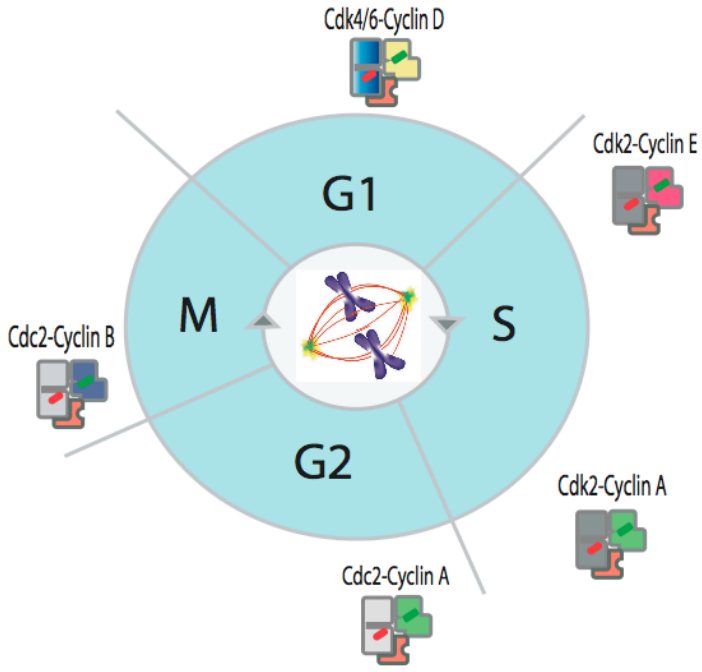

Figure 1. The principle cyclin-CDK complexes in cell cycle.

In most higher eukaryotic cells, the nuclear envelope is broken early in mitosis and re-forms around the separated chromosomes as they are decondensed during the last mitotic stage, telophase. Two daughter cells are produced by physical division of cytoplasm which is called cytokinesis. Following mitosis, cells enter G1 phase and reinitiate cycling into next S phase. In the absence of mitogenic signals, cells can quit cell cycle and enter into a non-proliferation phase like quiescence (G0 phase) or differentiation. These cells do not have duplicated DNA content and quiescence cells generally have reduced metabolic activity. In the response of various stimuli, certain quiescence cells can enter G1 phase and restart active division cycle.

B. Molecular bases of cell cycle progression

A number of controls not only operate strict regulation of cellular proliferation, but also make a decision in different phases of cell cycle to progress. Certain controls reflect the action of growth factors, hormones, extracellular matrix or direct the cell to growth, cell division, differentiation or cellular suicide (programmed cell death or apoptosis).

1. Cyclin and CDK

For cell proliferation, cells require enzymatic activity which has two alternating intertwined mechanisms, and this activity is called cyclin dependent kinase (CDK) (Morgan, 1997). CDK is a serine/threonine kinase and leads to the protein degradation periodically. CDKs consist of the family of homogenous size proteins (33-35kDa). They are inactive in monomeric form and associates with their regulatory subunits, cyclins in order to be active. The size of cyclins is various between 35 and 90 KDa in mammals. These regulatory subunits, whose intracellular concentration varies in different phases of cell cycle, control the sequential activation of CDK to guide the replication of DNA and cell division (Table 1). CDK activity oscillates through cell cycle and also requires precise coordination and regulation. The temporal and spatial controlling of CDK activity is critical in cell cycle progression and proliferation (Figure 1). The CDK activity is regulated positively or negatively by their temporal association with cyclins and CDK inhibitors (CKI), respectively. Although the level of CDK expression is constant throughout the cell cycle, the levels of cyclin and CKI are regulated by both transcriptional and post-translational processes. Generally, the concentration of cyclins results from the equilibrium between the regulation of transcription and degradation by ubiquitin dependent proteolysis (Murray, 1995; King et al., 1996; Koepp et al., 1999). In yeast, only one CDK, named Cdc2 in fission yeast *S. Pombe* and Cdc28 in budding yeast *S. Cerevisiae*, is capable of regulating all the events of cell division. The $p34^{cdc2}$ kinase (also known as Cdc2), the first known CDK in vertebrates (*Xenopus laevis*) was described as a catalytic subunit of M-phase promoting factor (MPF), a universal inducer of mitosis (Nurse, 1990). The activation of MPF induces mitosis and its inactivation permits the cell to exit mitosis and to enter interphase. In fact, only two identified CDKs, Cdc2 (renamed as Cdk1) and Cdk2, showing homologue functions of Cdc2 and Cdc28

kinases in yeast, control fundamental mechanisms of which DNA replication and mitosis. Certain CDKs have secondary roles in cell cycle or have no function in the regulation of cycle. In higher eukaryotes, Cdk4 and Cdk6 kinases permit the condition for entering cell cycle in the presence of extracellular mitogenic signals such as growth factors, cell adhesion and extracellular matrix (Sherr, 1996; Morgan, 1997).

Table 1. Principle CDKs implicated in cell cycle and their associated cyclins in vertebrates.

CDK	Associated cyclin		Principle function of complex
	Cyclin	Expression peak	
Cdk1 (Cdc2)	Cyclin A	S/G2/M	Transition G2/M
	Cyclin B1	G2/M	
	Cyclin B2	G2/M	
	Cyclin B3	G2/M	
Cdk2	Cyclin A	S/G2/M	Transition G1/S and S phase
	Cyclin B3	G2/M	Transition G2/M
	Cyclin E	G1/S	Transition G1/S
Cdk3	Cyclin E2		Transition G1/S
Cdk4	Cyclin D1	G1	G1 Regulation and Transition G1/S
	Cyclin D2	G1	
	Cyclin D3	constant	
Cdk5	Cyclin G	G2/M	Transition G2/M and role in apoptosis
Cdk6	Cyclin D1	G1	G1 Regulation and Transition G1/S
	Cyclin D2	G1	
	Cyclin D3	constant	
Cdk7 (MO15)	Cyclin H		CDK activating kinase (CAK), transcription

2. Mechanisms of CDK regulation

The mechanisms modulating CDK activity play a fundamental role in the transition of different phases through the cell cycle.

2-1. Activation of CDK

Besides the association with cyclins, most CDKs require the phosphorylation on the conserved Thr residue (Thr 160 in human Cdk2 and Thr 161 in human Cdk1) by CAK (CDK-Activating Kinase) for its activation. The activity of CAK is maintained at a high level through the cell cycle and phosphorylation of CDKs occurs only after the binding of cyclin to CDK in mammalian cells. However, little is known about how this

phosphorylation is regulated in cell cycle control pathway. The CDK activity can be restricted by the inhibitory phosphorylation on conserved Tyr (Tyr 15) and Thr (Thr 14) residues (Morgan, 1997). The phosphorylation of these two sites is assured by Wee1 (for Tyr 15) and Myt1 (for Tyr 15 and Thr 14) kinases.

2-2. The brake of cell cycle : CKI

The negative regulation of CDK is also achieved by CKI, which binds and inhibits the activity of cyclin-CDK complex (Elledge and Harper 1994; Sherr and Roberts 1995, 1999; Ekholm and Reed 2000). Until now, mammalian CKIs are classified in two families according to their sequence homologies, structural characteristics and activities.

a. INK family

INK4 (inhibitor of Cdk4) family members consist of four proteins; $p16^{INKA}$ (Serrano et al., 1993), $p15^{INKB}$ (Hannon and Beach 1994), $p18^{INKC}$ (Guan et al., 1994; Hirai et al., 1995) and $p19^{INKD}$ (Chan et al., 1995; Hirai et al., 1995). INK4 inhibitors contain an ankyrin repeat motif and specifically bind to Cdk4 and Cdk6. INK4 family members suppress the kinase activity by interfering with the formation of cyclins-CDK.

b. CIP/KIP family

The family members of Cip1/Kip1 $p21^{WAF-1/Cip1}$ (el-Deiry et al., 1993; Harper et al., 1993), $p27^{Kip1}$ (Polyak et al., 1994; Toyoshima and Hunter, 1994) and $p57^{kip2}$ (Matsuoka et al., 1995) contain a conserved sequence homology in their amino-terminals for CDK inhibitory domain. They inhibit the activity of a broad range of CDKs (Cdk2, Cdk4, Cdk6 and probably Cdk1) by binding to active cyclin-CDK complex.

c. p21 and cell cycle

i. Transcriptional regulation

p21 was discovered on the basis of its activation via a transcriptional factor, p53. However, the expression of p21 through p53 is only found in particular case such as genotoxic stress response. In human fibroblasts, the level of p21 mRNA is dependent on cell cycle phase. p21 mRNA expression reaches its peak in G1 phase (it is undetectable in quiescence cells) and decreases in S/G2 transition (Li et al., 1994). The expression of p21 can be induced during cell growth and differentiation under many circumstances, which reflects its numerous roles in cell cycle control (Parker et al., 1995; Macleod et al., 1995). Many cellular signals are capable of inducing the transcription of p21

independently of p53 such as transformation factor TGFβ (cytokine), protein kinase C, STAT, MAP kinase and cell adhesion events.

ii. Role of p21 in G1/S transition

Cyclin D-Cdk4/6 complexes associate with Cip/Kip family (mostly p21 and p27). The association of 'free' p21 and p27 with newly formed Cyclin D-kinase complexes helps to sequester Cip/Kip inhibitors from Cyclin E-Cdk2 complexes and allow their activation in late G1 (Sherr and Roberts, 1999; Ekholm and Reed, 2000). Activated Cyclin E-Cdk2 complexes amplify mitogen-induced signal pathway and facilitate the sequential phosphorylation of Rb (its functions will be discussed later). The importance of this CKI exchange for the strict control of cell cycle progression has been emerged by the elucidation of the role of proto-oncogene *c-myc* in G1/S transition. c-Myc is transcriptional factor whose expression is rapidly induced by mitogens. Cyclin D1, D2 and E as well as Cdc25A are known as transcriptional targets of c-Myc. The expression of c-Myc principally activates Cyclin E-Cdk2 complexes. This activation is the result of CKI exchange with D-type cyclins via the synthesis of cyclins. Hence the expression of c-Myc in Cyclin $D^{-/-}$ cells does not show full activation of Cyclin E-Cdk2 complexes (Bouchard et al., 1999; Perez-Roger et al., 1999). In Cdk4 disrupted cells, the efficiency of CKI exchange is diminished and cells show delayed S-phase entry (Tsutsui et al., 1999).

The level of 'free' p21 (not associated with Cyclin-CDK complexes) increases in G1 and decreases as cells approach S phase while the quantity of Cyclin A is elevated in S phase. This phenomenon induces the excess Cyclin A-Cdk2 complexes and facilitates G1/S transition when the concentration of Cyclin A-Cdk2 complexes exceeds that of p21. The fact that Cip/Kip inhibitors are re-distributed to the cyclin D complexes looks paradoxal in the context of their functional role in Rb phosphorylation. Nevertheless, many studies have shown that Cyclin D-Cdk4 complexes associate with Cip/Kip inhibitors in proliferating cells (Zhang et al., 1994). In fact, Cip/Kip inhibitors are relatively ineffective vis-à-vis with respect to these kinases (Blain et al., 1997). *In vitro*, p27 is capable of inhibiting Cyclin D-Cdk4 complexes, but its antagonistic effect is more effective on Cyclin E-Cdk2 complexes. Furthermore, interestingly, the function of p21 is regulated by its final concentration in cells. Low levels of p21 initially stimulate the assembly of cyclin-CDK complex *in vivo* to promote cell cycle progression and its accumulation in the nuclear whereas high levels of p21 inhibit cyclin-CDK activity. One report observed that the activity of D-type cyclin dependent kinases (Cdk4 and Cdk6) was significantly low in p21/p27 lacking MEFs (Mouse Embryonic Fibroblasts), which implies that p21 and p27 play an essential role in stimulating the assembly of Cyclin D-CDKs (Sugimoto et al., 2002).

iii. p21 and DNA replication

The N-terminal of p21 (residues 1-82) can inhibit DNA synthesis and cell growth by inhibiting CDK activity (Harper et al., 1995; Chen et al., 1995; Luo et al., 1995). The C-

terminal of p21 (residues 114-164) binds to PCNA (Proliferating Cell Nuclear Antigen) (Waga et al., 1994; Nakanishi et al., 1995; Boulaire et al., 2000; Dotto, 2000). p21 regulates PCNA activity by competitively inhibiting its interaction with RFC (replication factor C), DNA polymerase δ (Podust et al., 1995; Waga et al., 1998) and FEN1 (Flap endonuclease 1) (Chen et al., 1996). This inhibition is mediated through the repression of DNA polymerase δ or RFC ATPase but is not due to PCNA loading on DNA by RFC (Oku et al., 1998). Furthermore, p21 is also implicated in nucleotide excision repair (NER) through its interaction with PCNA. Thus, it has been reported that PCNA binding to C-terminus of p21 inhibits NER both *in vitro* and *in vivo*, and p21 regulates differentially DNA replication and repair according to its concentration for PCNA binding (Pan et al., 1995; Cooper et al., 1999).

iv. Role of p21 in cell cycle arrest induced by DNA damage

When DNA is damaged, cell cycle progression is blocked to facilitate the DNA repair. In fact, the inhibition of replication limits the propagation of potentially dangerous mutations. The tumor suppressor protein, p53 is involved in genomic stability and its inactivation is frequently seen in a number of cancer cells (Prives and Hall, 1999; Vogelstein et al., 2000). After DNA damage, p53 is stabilized and activated (Ko and Prives, 1996; Tibbetts et al., 1999). p53 induces the transcription of a number of genes including p21. The promoter of p21 possesses two p53 recognition sites, located 1.95 and 2.85 kb from p21 mRNA initiation site. Cell cycle arrest in G1/S transition after DNA damage is due to the accumulation of p21 (Kastan et al., 1991; Kuerbitz et al., 1992; Lu and Lane, 1993; el-Deiry et al., 1994). In this case, p21 inhibits the kinase activity associated with Cdk2 (Dulic et al., 1994). p21-deficient cells are partially (or completely) incapable of blocking their cell cycle in G1 phase after DNA damage (Brugarolas et al., 1995; Deng et al., 1995; Waldman et al., 1995). The principal consequence of CDK associated kinase activity inhibition is the accumulation of hypophosphorylated Rb which is associated to E2F (Slebos et al., 1994; Harrington et al., 1998). Thus, Rb fails to be phosphorylated by Cdk2 after DNA damage in wild type cell, but not in p21-deficient cell (Brugarolas et al., 1999). Rb-deficient MEFs do not arrest in G1 after DNA damage despite increased level of p53 and p21 in these cells (Harrington et al., 1998). The role of p21 in G2 arrest after DNA damage still remains unclear. However, it has been reported that p21 is essential for maintaining G2 arrest following DNA damage in human cells (Beamish et al., 1996; Waldman et al., 1996; Bunz et al., 1998). p21 may play a role in the onset of mitosis through its accumulation in the nuclear and Cdk1 inhibition at G2/M transition (Dulic et al., 1998), but not in maintaining G2 arrest for sufficiently long time to repair DNA before entering the mitosis (Andreassen et al., 2001). The G2 arrest mostly occurs through p21-mediated inhibition of Cyclin A-Cdk2 activity and this inhibition leads to inhibitory phosphorylation of Cdc2 concomitantly with G2 arrest (Guadagno et al., 1996). Moreover, several studies described that the p21-induced inactivation of CDKs blocks transcription of a number of genes involved in G2/M progression through Rb dephosphorylation (Taylor et al., 2001; Ren et al., 2002).

v. The regulation of p21 turnover in cell cycle

p21 is a target for ubiquitin-proteasome dependent proteolysis. Several studies show that the genotoxic stress (γ-irradiation or UV) appears to be not involved in the stability or *in vivo* ubiquitination of p21 (Blagosklonny et al., 1996; Maki and Howley, 1997). In contrast, low (but not high) dose of UV irradiation can induce the Skp2-dependent degradation of 21 (Bendjennat et al., 2003). It has been also reported that the SCF^{Skp2} is likely to function in the degradation of p21 during the G1/S transition (Yu et al., 1998) and Skp2-deficient MEFs show the delay of p21 degradation (and an increase in p21 half-life) in the S phase (Bornstein et al., 2003). Surprisingly, a very recent report proposed an alternative degradation pathway in which the proteolysis of p21 mediated by APC^{Cdc20} for its positive feedback role in Cdk1 activation in early mitosis (Amador et al., 2007). In addition, it is of interest that the ubiquitination on internal lysine residues or on N-terminus is not essential for p21 turnover (Sheaff et al., 2000; Bloom et al., 2003; Chen et al., 2004). A direct interaction between p21 and 20S proteasome for degradation is also reported (Touitou et al., 2001). Thus, it is likely that various degradation pathways are implicated in p21 turnover.

3. Regulation and control of cell cycle progression

a. Regulation of G1 to S phase progression

After each cell division, cells must make a decision to initiate a new cycle of DNA replication or to choose an alternative such as differentiation or quiescence. This decision occurs slowly in G1 and is termed as a restriction point (Pardee, 1989; Zetterberg et al., 1995). Once the cell reaches this restriction point, it is committed to complete the cycle starting with DNA replication in S phase to M phase. To reach this restriction point, cell must be stimulated continuously by mitogenic factors. The restriction point passage and the entry to S phase are controlled by susceptibility of retinoblastoma protein, (p)Rb (Weinberg, 1995). Rb is a negative regulator of cellular proliferation. Cell cycle progression and its decision to enter S phase are dependent on its phosphorylation state. Rb is present in hypophosphorylated form in G0 and early G1 whereas Rb is observed in hyperphosphorylated form in all the rest of cell cycle until the end of mitosis. Rb is referred to as a pocket protein because they contain an E2F-binding pocket. The phosphorylation of Rb in G1 phase, which induces the dissociation of binding between pRb and E2F, can activate the transcription of necessary genes for the entry and progression into S phase (Figure 2). E2F proteins activate gene expression and its activity is repressed by hypophosphorylated Rb. The five major human E2F proteins are divided into two functional groups; E2F1-3 stimulate G1/S gene expression and thus promote cell cycle entry. E2F4-5 interact with p107 and p130, which are pRb-related proteins, to actively inhibit G1/S gene expression during G0 and G1 (Mulligan and Jacks, 1998; Harbour and Dean, 2000). In general, Rb can bind to E2F1-4.

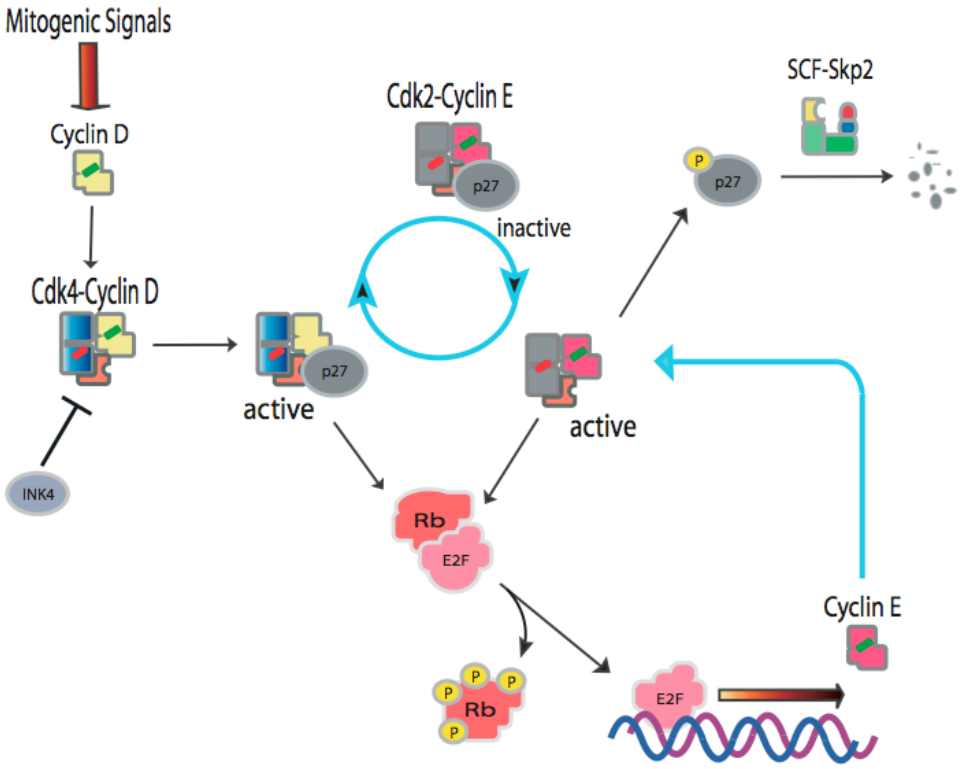

Figure 2. Regulation of G1/S transition.

Mitogenic signals induce Cyclin D synthesis and Cyclin D-Cdk4/6 complex assembly associated with Cip/Kip protein, $p27^{Kip1}$, which relieves Cyclin E/Cdk2 from their constraint. Both G1 cyclin-dependent kinases sequentially phosphorylate Rb family proteins to liberate E2F transcriptional factor and leads to the activation of genes required for S phase entry. Among E2F target genes, transcriptional upregulation of Cyclin E is providing for positive feedback to drive cells into S phase. The phosphorylation of p27 by Cyclin E-Cdk2 triggers its ubiquitination and degradation. p27 proteolysis contributes to reduce the requirement of mitogenic factors and to make the transition irreversible (Adapted from Sherr and McCormick, 2002).

In many mammalian cell types, complete E2F activation is achieved through the sequential phosphorylation of Rb by Cyclin D-Cdk4/6 and Cyclin E-Cdk2 (Sherr and Roberts, 1999; Stevaux and Dyson, 2002). In addition, active Cyclin E-Cdk2 complexes phosphorylate p27 protein and induce its degradation by proteosome pathway (Pagano et al., 1995; Sheaff et al., 1997; Vlach et al., 1997). Thus, the increased levels of Rb phosphorylation and E2F dependent transcriptional activity contribute to irreversible passage of restriction point.

b. Regulation of DNA synthesis in S phase

DNA replicative initiation process is regulated by Cdk2 in association with two different regulatory subunits, Cyclin A and Cyclin E (Sherr and Roberts, 1999). Cyclin E stimulates replication complex assembly on chromatin, and Cyclin A activates DNA synthesis and it prevents assembly of new complex before DNA synthesis begins in order to inhibit re-initiation until the next cell cycle (Coverley et al., 2002). Thus, Cyclin E makes the cell start DNA replication with assembly of new complex, and Cyclin A ends its DNA replication process. Cdk2 is thought to regulate entry into S phase, whereas Cdk1 controls the initiation of mitosis. However, in contrast with classic dogma, recent reports showed that Cdk2 knockout mouse has no developmental or cell cycle abnormalities and Cdk2 is not essential for mitotic cell division (Ortega et al., 2003; Roberts and Sherr, 2003; Sherr and Roberts, 2004). Cdk1 can equally promote the G1/S transition when Cdk2 activity is compromised (Bashir et al., 2005), and Cdk1 alone is sufficient for all the events to drive mammalian cell cycle in the absence of other interphase CDKs (Santamaria et al., 2007). Moreover, Cyclin E can bind to and activate Cdk1 for promoting G1/S transition and Cyclin E-Cdk1 may constitute the loss of Cdk2 function in mice (Aleem et al., 2005; Bashir and Pagano, 2005). Surprisingly, one study showed that Cyclin E is associated with chromatin and promotes DNA replication licensing in a CDK-independent manner (Geng et al., 2007; Zhang, 2007).

Cyclin A-Cdk2 complexes appear to phosphorylate Rb in S phase and contribute to maintenance of Rb inactivation during S-G2 progression and mitosis until Rb is dephosphorylated by the phophatase protein type 1 (PP1) (Mittnacht, 1998; Rubin et al., 2001). The importance of Rb in the regulation of DNA synthesis has been reported by showing that the recruitment of elongation factors to chromatin is inhibited or requisite replication factors are downregulated in S phase (Angus et al., 2004).

Initiation of DNA replication requires the integration of two central processes: (1) formation of prereplicative complex (pre-RC), namely 'licensing' for DNA replication and (2) activation of DNA-unwinding and polymerase functions. The former can occur only when CDK activity is low, whereas the latter is promoted when CDK activity is high (Dahmann et al., 1995; Diffley 1996, 2001; Piatti et al., 1996; Noton and Diffley 2000). The key player in the assembly of the pre-RC is the origin recognition complex (ORC). The formation of the pre-RC requires two other proteins, Cdc6 and Cdt1, which is associated with ORC and they recruit MCM complex. A protein called 'geminin', which binds to Cdt1 and prevents it from binding to the ORC, can inhibit the assembly of pre-RC. Geminin is accumulated in late G1 and binds to Cdt1 from S phase to mitosis. When the initiation of DNA synthesis is triggered in early S phase, highly activated CDKs promote the destruction or inhibit individual pre-RC component to prevent from its re-assembly. In this way each mitotic cycle has only a single DNA replication (Donaldson and Blow, 1999). The activation of replication origin not only requires CDK, but also a second protein kinase, Cdc7, which is direct activator of origin firing. Cdc7 is associated with a regulatory subunit, Dbf4. The activity of Cdc7 is changed along with the level of Dbf4, which is increased in late G1 and remains high until the exit from mitosis. Dbf4 is thought to be targeted and degraded by APC^{Cdh1} during G1, and its degradation leads to inactivation of Cdc7. Cdc7-Dbf4 complexes phosphorylate MCM subunits, which have DNA helicase activity, at activated replication origins. The activation of CDKs and Cdc7 promote the formation of the preinitiation complex for the initiation of DNA replication. The preinitiation complex activates the MCM helicases, recruits RPA onto the single-

strand DNA to prevent its reannealing and loads DNA polymerases onto the origin (Walter and Newport, 2000; Bell and Dutta, 2002) (Figure 3). The DNA replication must be complete before chromosome separation is triggered. However, if DNA replication fails during S phase, a regulatory system detects the DNA damage in stalled replication forks and sends a signal to block the firing of other replication origins and prevents entry into mitosis.

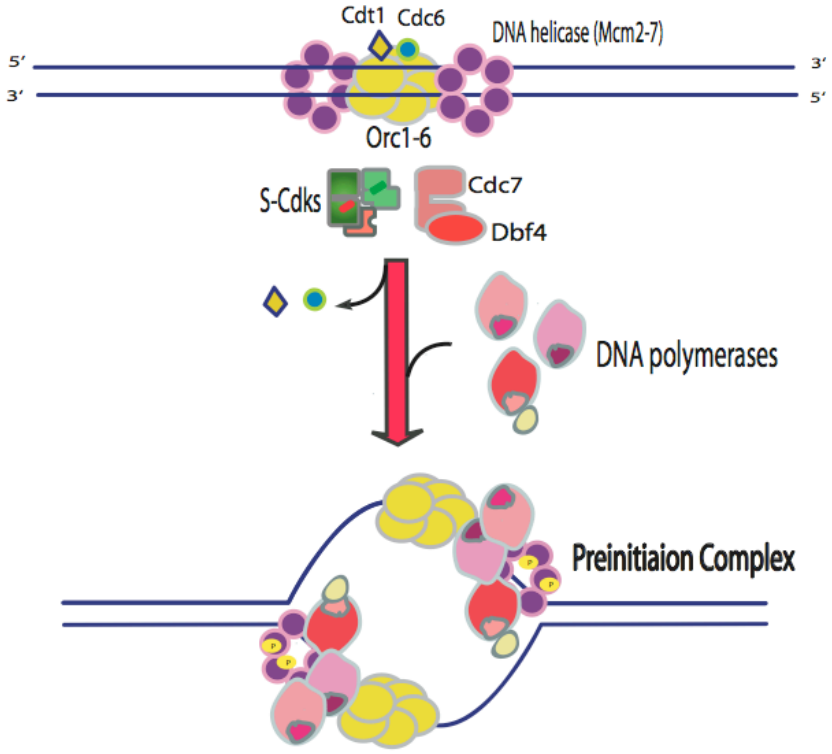

Figure 3. Initiation of DNA replication.

The pre-RC is assembled to the replication origin for firing during G1. S-CDKs and Cdc7 trigger the origin activation to promote the formation of the preinitiation complex with DNA polymerases and other core components (not shown). Cdt1 and Cdc6 are detached from ORC and Preinitiation complex activates MCM helicase for unwinding the DNA helix (Adapted from the text book, The cell cycle; Principles of control. David O Mogan, 2007).

c. Polyploidy

Uncoupled S phase and mitosis can induce chromosomal instability, which results in grossly deformed, polyploidy and apoptosis. The additional S phases without intervening normal mitosis is called endoreplication or rereplication. Endoreplication gives rise to cells with extra copies of the genomic DNA. In endoreplicating cell cycle, S phases are

alternated with distinct gap phases, but there is no cell division. Continuous DNA replication can cause polyploidy and many cases shows that endocycling cell lacks of traces in mitosis, for example, such as chromosome condensation, nuclear envelope breakdown, and the reorganization of microtubules (Mahowald et al., 1979). DNA rereplication in cell cycle may cause significant genetic instability, which shows chromosomal translocations, mircrosatellite instability, gene amplications, and aneuploidy in human cancer (Lengauer et al., 1998).

Three possible mechanisms might render cells polyploid.

Failure of mitosis (or Endomitosis)

~ Cells enter mitosis normally but anaphase and cytokinesis fail to occur, resulting in the subsequent entry of the cells into interphase with a doubled DNA content.

Endoreplication

~ Cells replicate their genomes in S phase, bypass mitosis, and double their DNA content again in the next S phase.

DNA rereplication

~ Cells arrest in S phase and reinitiate DNA replication continuously.

Endoreplicated cells appear to show simplified cell cycle by removing unnecessary components. For instance, some cell types which bypass mitosis show lowered level of Cdk1 activity or its activators, cyclin B, cyclin A and Cdc25C. Thus, premature degradation of cyclin B or abnormal activity of Cdk1 can cause the mitotic exit prior to late anaphase, nuclear division, and cytokinesis (Vitrat et al., 1998). p53-dependent accumulation of p21 in the response of DNA damage (for example, ionizing radiation) can cause cell cycle arrest. In the absence of p21, DNA damaged cells were shown to delay in G2 and M, but then undergo additional S phases without normal mitosis leading to gross nuclear abnormalities and culminating in apoptosis (Waldman et al., 1996). Functional Rb is necessary to prevent DNA replication in p21-mediated G2 arrest cells and the Rb-negative cells can undergo endoreplicating cycles without mitosis. After arrest in G2, a significant subpopulation of Rb-negative cells enter endoreplicative DNA replication cycle in response to p21 or p27 expression, while endoreplication is not observed in Rb-positive cells arrested in G2 (Niculescu et al., 1998). Polyploidy can be induced after the treatment of microtubule inhibitors (MTIs). p21-deficient cells re-enter S phase for replication after aberrant mitotic exit, which occurs in long-term MTI treatment, and MTI-induced polyploidy is mediated by cyclin E and Cdk2 activity through the regulation of p21 (Stewart et al., 1999).

Geminin is a inhibitor of replicative initiation factor, Cdt1. However, interestingly, geminin-Cdt1 complex is required for the stabilization of Cdt1 during mitosis, thereby allowing sufficient accumulation of Cdt1 for pre-RC formation (Ballabeni et al., 2004). Geminin-Cdt1 complex allows to load the MCM2-7 helicase onto chromatin for origin firing (Lutzmann et al., 2006). Cdk1 inhibition in G2 induces the rebinding of Cdt1 on

the chromatin independently of geminin binding in murine cells and the overexpression of Cdt1 with Cdc6 during G2 can also induce relicensing and lead rereplication (Vaziri et al., 2003; Sugimoto et al., 2004; Maiorano et al., 2005). Furthermore, It has been reported that transient knockdown of geminin can induce rereplication in human cell lines (Melixetian et al., 2004; Zhu et al., 2004), and endoreplication is increased in geminin-deficient mice during early embryogenesis of trophoblasts (Gonzalez et al., 2006). These results suggest that geminin is required prior to Cdt1 degradation to prevent rereplication because abundant Cdt1 can induce rereplication in the absence of geminin (Arias and Walter, 2005; Li and Blow, 2005; Yoshida et al., 2005).

Recent studies propose that the depletion of APC (Anaphase-Promoting Complex, APC will be discussed later) inhibitor, Emi1, can cause rereplication (Machida and Dutta, 2007). In the absence of Emi1, unscheduled activation of APC^{Cdh1} may be involved in the degradation of geminin and cyclin A after mitosis, which results in rereplication. In addition, co-depletion of geminin with Cdk1 and Cdk2 induces rereplication, implying that Cdk1/2 activities are involved in prevention of rereplication. However, another study proposed that the increased level of cyclin E and Cdk2 activity are implicated in Emi1 depletion induced rereplication (Di Fiore and Pines, 2007).

In conclusion, the balance between CDK and APC activities in cell cycle may further secure from inappropriate endoreplication. Pre-RC is assembled for replication licensing during mitosis and early G1 when CDK activity is low, and APC activity keeps CDK inactive by destruction of its cyclins during mitosis. In S and G2 phase, high Cdk2 and Cdk1 activity promotes DNA replication and inhibits pre-RC formation by preventing re-accumulation of Cdc6 and MCM proteins (relicensing). This mechanism ensures that cells couple DNA replication with mitosis in each cell cycle.

d. Regulation of M phase entry

Once DNA replication is completed, cells prepare the necessary events for the mitotic entry and progression during G2 phase. In this phase, cyclin B accumulates and associates with Cdk1 (Cdc2) to control mitotic entry (Nurse, 1990). Cyclin B-Cdk1 is phosphorylated on Thr161 by CAK and maintained in inactive form by Wee1 and Myt1 kinases which phosphorylate on Thr14 and Tyr15 of Cdk1. Both activities of Wee1 and Myt1 are high during most of cell cycle but decreased during mitosis. Dephosphorylation of Thr14 and Tyr15 of Cdk1 occurs rapidly in the end of G2, which is catalyzed by the B and C isoforms of Cdc25 phosphatase. Cdc25 induces the final activation of Cdk1 and triggers the mitotic entry (Morgan, 1997). The activity of Cdc25B is thought to be high in late S and G2, peak in prophase for the initiation of Cdk1 activation and decreases in prometaphase. Cdc25A and Cdc25C are relatively inactivated in G2 but their activities increases in prophase. The level of Cdc25C does not change during cell cycle, but it seems that its catalytic activity is increased in mitosis. Cdc25A is located mostly in the nucleus whereas Cdc25C is in the cytoplasm in early prophase and moved into the nucleus in late prophase. Therefore, rapid Cdk1 activation in prophase is due to the increase of Cdc25A and Cdc25C activities, combined with simultaneous decreases in the activities of Wee1 and Myt1.

Positive feedback for ensuring complete and irreversible mitotic initiation lies at the heart of mitotic Cdk1 activation. Cdk1 phosphorylates and thereby stabilizes the Cdc25A protein and the phosphorylation of Cdc25C stimulates enzymatic activity of Cdk1 (Mailand et al., 2002). Wee1 and Myt1 can be also phosphorylated and inhibited by Cdk1 (Harvey et al., 2005). Another mitotic serine/threonine kinase called polo-like kinase (Plk) is involved in this positive feedback loop additionally and it seems to be stimulated by Cdk1. Cyclin A-CDK can also help to trigger Cyclin B-Cdk1 in late G2 until cyclin A is degraded in prometaphase by phosphorylating Cdc25A, Cdc25C, Myt1 or Wee1 (Figure 4). Consistent with this hypothesis, the inhibition of Cyclin A-Cdk2 delays mitotic entry through Cyclin B-Cdk1 activation in human cells (Mitra and Enders, 2004). Cyclin B-Cdk1 complexes are located in cytoplasm during G2, and rapidly imported into the nuclear just before the nuclear envelope breakdown. The activation of Cdc25C and the nuclear accumulation of Cyclin B-Cdk1 are thought to be triggered in early mitosis by Cdk1 and Plk activities. The Cdk1 activation leads the phosphorylation of a number of substrates which catalyze morphologic and molecular changes during mitosis (Nigg, 1993). For instance, Cyclin B-Cdk1 complexes phosphorylate the proteins involved in DNA condensation (Histone H-1, Condensin complex) (Murray, 1998; Kimura et al., 1998), nuclear envelope disassembly (Lamine A, B, and C) (Nigg,1992), microtubule dynamics and mitotic spindle formation (MAPs, Stathmine) (McNally, 1996 ; Andersen et al., 1997).

e. Regulation of mitotic progress

Once the chromosome separation is permitted, mitotic spindle is assembled and nuclear envelope is broken in prometaphase. Each chromatid carries a kinetochore at the beginning of mitosis (Pluta et al., 1995) to which is captured by microtubules of mitotic spindle. The sister chromatids are linked each other until their final separation in anaphase. The cohesion between chromatids is assured by a protein complex named 'Cohesin', which is conserved both in yeast and vertebrate (Koshland and Guacci, 2000) and placed during DNA replication. The cohesin links between sister chromatids are abruptly dissolved at the metaphase-anaphase transition and each sister chromatid is pulled to opposite poles of spindle. The destruction of securin releases the active separin protease (also known as separase), allowing it to cleave proteins mediating sister chromatid cohesin (Glotzer, 1999; Uhlmann et al., 1999; Nasmyth et al., 2000; Uhlmann et al., 2000; Waizenegger et al., 2000). Complete separation of sister chromatids occurs in telophase, where the chromosomes and other nuclear components are re-located in daughter nuclei. The spindles are disassembled and a nuclear envelope reforms around the decondensing chromosomes. Mitotic progression requires several other protein kinases. The most important mitotic kinases are polo-like kinase 1, Plk1, and two protein kinases named Aurora A and Aurora B. Plk1 is thought to be activated by Cdk1 in early mitosis, but its mechanism of activation remains unclear. The functions of Plk1 lies in particular in chromosome separation, spindle assembly and cytokinesis (Nigg, 1998) and they are involved in centrosome amplication and maturation (Liu and Erikson, 2002). Another mitotic kinase group is aurora family. Similar with Plk1, aurora kinases are also activated in mitosis. Aurora A is located at the centrosome and on the spindle, and it

functions in controlling bipolar spindle assembly and stability. Aurora B is found in early mitosis on condensing chromosome arm, and helps to stimulate chromosome condensation and control kinetochore attachment to the spindle (Shannon and Salmon, 2002). The inhibition of aurora B can often lead to the failure of cytokinesis (Kallio et al., 2002).

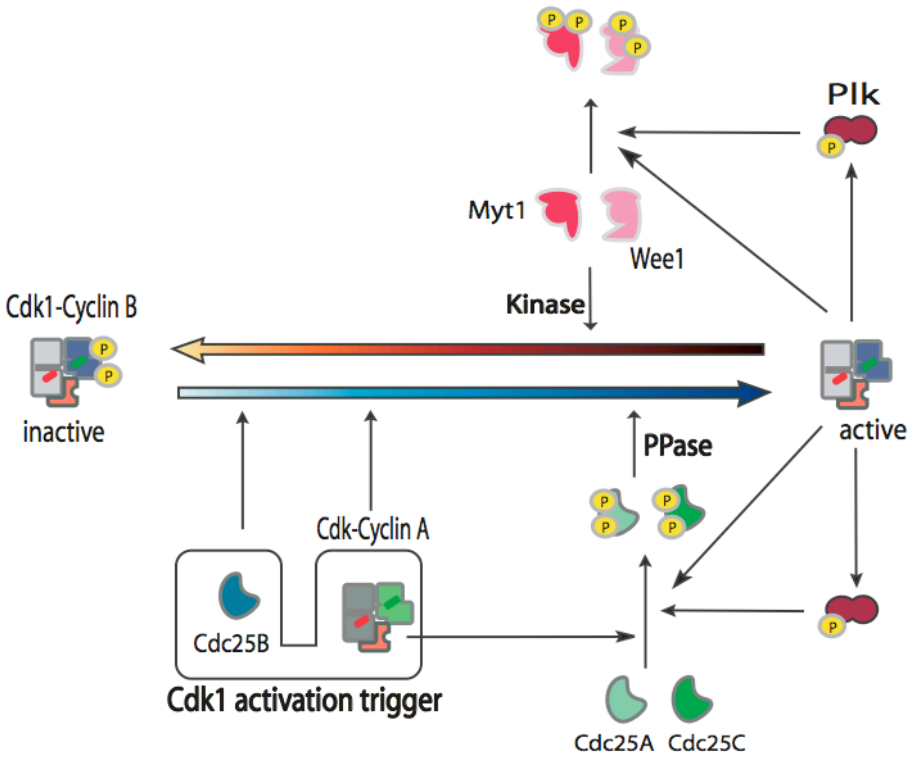

Figure 4. Regulation of Cdk1 activation in early mitosis.

Cdc25B and cyclin A-CDK may help to trigger Cdk1 activation by partial dephosphorylation in late G2. The complete activation of Cdk1 is achieved by two phosphatase Cdc25A and Cdc25C. Plk is activated by Cdk1 and both phosphorylate Cdc25A and Cdc25C for positive feedback loop. The phosphorylation of Wee1 and Myt1, which are inhibitory kinases of Cdk1, requires Cdk1 and Plk activities for their negative regulation (Adapted from the textbook, The cell cycle; Principles of control. David O Morgan, 2007).

C. Illustration of two checkpoints in cell cycle

1. DNA damage checkpoint

The living organism must distribute equally their chromosomes with minimal mutation. To achieve this fidelity, surveillance mechanisms are required for monitoring the structure of chromosomes and cell cycle progression. Upon DNA is damaged, the

damage sensors trigger a 'DNA damage response'. This signaling pathway triggers DNA repair systems or block cell cycle progression through various effector proteins (Wang, 1998; Bartek and Lukas, 2001) (Table 2). If the damage is repaired, the blocked cell cycle is restored and cell proliferation continues. When the damage is unrepairable, control mechanisms eliminate the potentially dangerous cells by imposing permanent cell arrest or inducing cell death (apoptosis). Defects in the DNA damage response not only lead to impaired DNA repair but also chromosomal instability.

In all eukaryotes, the DNA damage response is related to a pair of protein kinases called ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3-related), whose sequences and functions have been well conserved in evolution (Zhou and Elledge, 2000). These two proteins are members of PI-3 Kinase family and activated in response to DNA damage. ATR is involved in many different types of DNA damage response, including nucleotide damage, stalled replication forks, and double-strand breaks while ATM is only specialized for the response to double-strand breaks. The ATR and ATM kinases control their target kinases (Checkpoint kinase), Chk1 and Chk2. This activation of cellular signal pathway can induce cell cycle arrest or slow down in DNA replication when DNA is damaged during S phase. It is likely that Chk1 has a essential role in mammalian development and its viability as Chk1 knock-out mice show early embryonic lethality unlike in Chk2-deficient mice (Takai et al., 2000). However, it is also reported that Chk1 is not required for normal somatic cell growth (Zachos et al., 2003). Among the targets of ATM/ATR-Chk1/Chk2 pathway, Cdc25 and p53 proteins play an important role in cell cycle arrest after DNA damage (Figure 5). The tumor suppressor protein p53 is another essential target in cell cycle arrest and maintenance of G1 phase. p53 is a transcriptional factor implicated in the regulation of a number of cellular responses such as cell growth arrest, DNA repair, and programmed cell death (apoptosis) (Levine, 1997).

1-1. DNA damage in G1

Recent studies indicate that one of targets of Chk1 and Chk2 kinases is Cdc25A phosphatase which plays a pivotal role in Cdk2 activation by dephosphorylating Thr14 and Tyr15 of kinase (Mailand et al., 2000; Costanzo et al., 2000). The persistence of inhibitory phosphorylation of Cdk2 on Tyr15 resulting from the loss of Cdc25A activity can keep Cyclin E-Cdk2 complexes inactive and block the G1/S transition. Chk1/Chk2 and ATM/ATR participate in phosphorylation of p53 for its stabilization and activation (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000; Zhang and Xiong, 2001). Stabilized p53 permits the transcription of target genes coding notably CDK inhibitor, p21 and other genes more directly implicated in DNA repair (Levine, 1997). The induction of p21 inhibits the activity of Cyclin E-Cdk2 complexes which is required for the G1/S transition and in consequence, maintenance of cell cycle arrest in G1 phase. In addition, p53 and p21 can limit Rb hyperphosphorylation and loss of Rb function can bypass p53-mediated G1 arrest (Demers et al., 1994).

1-2. DNA damage in S phase

The cell does not enter mitosis until DNA replication is completed successfully. DNA damage response on stalled replication folks sends inhibitory signals to stop entry into mitosis and to promote DNA repair. ATR binds and regulates ATRIP (ATR-interacting protein) through its phosphorylation, and both co-localize in intranuclear foci after DNA damage or replication block. ATRIP mutated cells show damage response defects and cause loss of ATR and ATRIP expression, which implicates the importance of ATRIP in ATR function (Cortez et al., 2001). Single-strand DNA binding protein, RPA (Replication Protein A) is recruited on DNA damaged site (Zou and Elledge, 2003) and ATR phosphorylates the target proteins such as p53, H2AX, and Chk1 (Liu et al., 2000; Tibbetts et al., 1999; Ward et al., 2001).

Table 2. Alternative names for DNA damage response components

		S. Cerevisiae	***S. Pombe***	**Vertebrates**
Sensor Kinases	ATR	Mec1	Rad3	ATR
	ATM	Tel1	Tel1	ATM
ATR Regulatory Subunit	ATRIP	Ddc2/Lcd1	Rad26	ATRIP
Effector Kinases	Chk1	Chk1	Chk1/Rad27	Chk1
	Chk2	Rad53	Cds1	Chk2

Claspin, Chk1 large associated protein, recruits the phosphorylated Chk1 by ATR onto the DNA lesions (Kumagai and Dunphy, 2000), and Chk1 is required for preventing unscheduled initiation of DNA replication in response to DNA damage (Feijoo et al., 2001). ATM binds to the MRN complex, which is composed of three proteins: Mre11, Rad50, and Nbs1, and is recruited to the site of damage (Lee and Paull, 2004). The activation of ATM may be dependent on autophosphorylation of the kinase (Lee and Paull, 2005) and active ATM phosphorylates target proteins.

Cdk2 is a key regulator in S phase. The inactivation of Cdk2 is one of main targets in the S phase DNA damage. In response to DNA damage, Chk1 and Chk2, in turn, inactivates Cdc25A. This prevents the dephosphorylation of Cdk2 for its activation and inhibits S phase progression (Zhao et al., 2002; Hu et al., 2001). Several studies showed that the degradation of Cdc25A in response to DNA damage during S phase inactivates Cdk2 and induces a delay in DNA replication and prevents premature mitosis (Falck et al., 2001; Molinari et al., 2000).

1-3. DNA damage in G2/M phase

The maintenance of inhibitory phosphorylation on Cdk1 plays a major role in cellular response to DNA damage during G2 phase, which blocks the G2/M transition. The mechanism of G2 arrest seems to implicate at least partially the inactivation and the

translocation of Cdc25C in cytoplasm (Zhou and Elledge, 2000). DNA damaged-induced activities of Chk1 and Chk2, in turn, are involved in the inactivation of Cdc25 phosphatase through its phosphorylation and degradation, thereby inhibiting CDK activity and causing cell cycle delays (Zhao et al., 2002; Hu et al., 2001). The phosphorylation at S216 of Cdc25C by Chk1 and Chk2 inhibits its phosphatase activity (Blasina et al., 1999) and makes binding site with 14-3-3 proteins (Peng et al., 1997). The binding between Cdc25C and 14-3-3 proteins induces the nuclear export of Cdc25 and its retention in the cytoplasm (Zhou and Elledge, 2000). In yeast, the sequestration of Cdc25C in the cytoplasm blocks the mitotic entry by preventing the activation of Cyclin B-Cdk1 complexes, which are located in nuclear (Lopez-Girona et al., 1999). In mammalian cells, Cyclin B-Cdk1 complexes remain in the cytoplasm after DNA damage (O'Connell et al., 2000). 14-3-3σ protein is induced after DNA damage and seems to be responsible for the sequestration of Cyclin B-Cdk1 complexes in the cytoplasm in response to DNA damage (Hermeking et al., 1997; Chan et al., 1999). Another main target of DNA damage in G2/M is Polo-like kinase 1 (Plk1) (Smits et al., 2000). DNA damage interferes with its phosphorylation on Threonine residue in T-loop for the inactivation of Plk1.

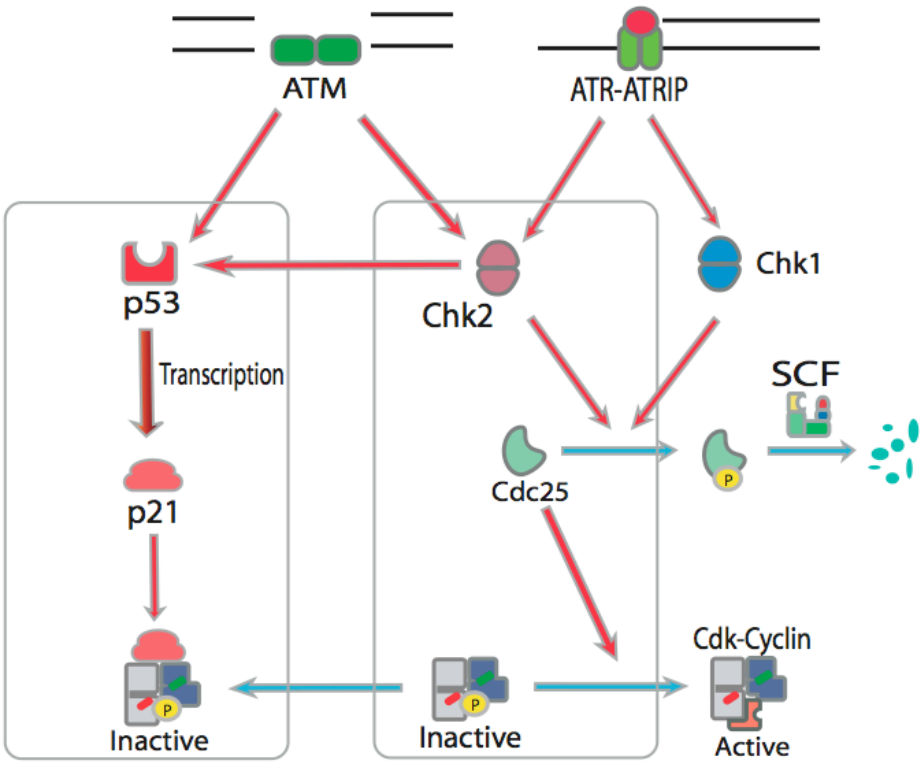

Figure 5. DNA damage response.

The damaged single-strand DNA recruits the protein kinase ATR, and ATM is required for the response to DNA double-strand breaks. ATR/ATM activate Chk1 or Chk2 respectively, which phosphorylates Cdc25,

targeting it for ubiquitination and degradation. As a result, the inhibitory phosphorylation of cyclin-CDK leads to inhibit cell cycle progression. ATM/Chk2 also stabilizes and activates p53, which increases the transcription of CDK inhibitor, p21. p21 inactivates CDKs and helps to maintain long-term cell cycle arrest (Adapted from the textbook, The cell cycle; Principles of control. David O Morgan, 2007).

2. Mitotic checkpoint

In late anaphase, the sister chromatids is separated and divided into the two halves. A defective cell division or chromosome segregation may lead to anueploid between diploid and tetraploid. Such an aneuploid is frequently observed in tumoral cells. Certain cancer cells show chromosome instability and it is associated with the dysfunction of mitotic checkpoints (Cahill et al., 1998). The onset of anaphase requires the kinetochore attachment of microtubule spindle. Even one single missed disjunction of kinetochore can lead to the delay of anaphase onset (Reider et al., 1994). In the presence of microtube depolymerizing drugs, the same mitotic arrest is observed. This mitotic checkpoint involved in the spindle formation is called <<spindle checkpoint>> or <<spindle assembly checkpoint>>; Three genes of MAD (Mitotic Arrest Dificient) which encodes Mad1, Mad2 and Mad3 (Li and Murray, 1991), two genes of BUB (Budding Uninhibited by Benomyl) which encodes Bub1 and Bub3 (Hoyt et al., 1991), and MPS1 gene (Monopolar Spindle) (Weiss and Winey, 1996). The homologs of Mad1, Mad2, Mad3, Bub1, Bub3, and Mps1 had been characterized in vertebrates (with the exception of Mad3 of which the ortholog is an hybrid between Mad3 and Bub1, called BubR1) and are implicated in mitotic checkpoint (Wassmann and Benzera, 2001; Abrieu et al., 2001). The spindle checkpoint proteins monitor the attachment of kinetochore with microtubule spindle. These proteins are concentrated near kinetochore during mitosis. In contrast, Mps1 is accumulated at maximum level when kinetochore is not attached to microtubules. The level of Mps1 is diminished gradually when kinetochores were captured and fixed by microtubules and aligned with metaphase plate (Shah and Cleveland, 2000; Abrieu et al., 2001).

The anaphase onset requires the activation of APC^{Cdc20} (Anaphase-Promoting Complex, APC will be discussed later). Among the spindle checkpoint proteins associated with kinetochore, Mad2 and BubR1 are susceptible of intervening APC activation. These proteins bind to and inhibit the activity of APC^{Cdc20} toward securin and mitotic cyclins, delaying their destruction until all sister chromatids are correctly aligned (Shah and Cleveland, 2000; Hoyt, 2001). In the absence of spindle checkpoint, securin and cyclin B are degraded prematurely in early mitosis, but it still remains unclear how spindle checkpoint delay the destruction of these APC targets while allowing the degradation of cyclin A. In mammalian cells, the process of chromosome segregation generally takes less than 1 hour. The spindle checkpoint can delay the anaphase onset for a few hours, but this delay is rarely permanent. During the process called « adaptation » or « mitotic slippage » of which the mechanism is unknown, the cell escapes from mitotic control mechanism and exit from mitosis, without its division. The cell enters G1 phase with 4N chromosomes instead of 2N (tetraploid cell in G1-like phase).

Deuxième Partie : La Voie de Degradation de l'Ubiquitine-Protéasome
<< Ubiquitine-proteosome degradation pathway >>

A. Ubiquitine-proteosome degradation pathway

Fundamental cellular functions such as DNA replication, mitosis, transcription, cell differentiation and cell death are strictly and precisely regulated. The cell cycle mechanisms are driven by crucial modular components – for example, the cyclin-dependent kinases, CDKs - and these driving forces must lock into place or detach and disappear in order to keep cell cycle coordination. The disappearance of components in a sudden and compartment-restricted manner can be finely tunable brake (if the target component is catalyst) or a sensitive accelerator (if the target component is inhibitory subunit). These regulated disappearances can be achieved by the ubiquitin-proteosome system. Ubiquitin is well-established protein in the view of phylogenetics. This protein consists of 76 amino acids (8.5 kDa), and is mostly involved in the post-translational modification for protein degradation. The proteolysis by ubiquitin-proteosome pathway functions in two steps. First, the substrate is marked covalently with ubiquitin chains by a specialized enzymatic cascade. The sulfhydryl group of a cystein in E1 enzyme forms a thiolester bond with Gly of ubiquitin. Ubiquitin-conjugating E2 (or Ubc) proteins transfer activated ubiquitin to substrate, with or without the intervention of one of numerous protein-ubiquitin ligases E3. Several rounds of ubiquitin conjugation can produce long chains of ubiquitin moieties (polyubiquitinaiton). The polyubiquitylated substrate is degraded by the 26S proteosome and there is specificity in substrate degradation (Hershko and Ciechanover, 1998). The combinatory interactions between different E2 and E3 generate a number of specific complexes of target substrates. The spatial and temporal destruction of specified substrates by Ubiquitin-proteosome pathway is constant with its role in division and cellular signalization control, transcription and development.

In ubiquitin-mediated proteolysis machinery, there are two distinct alternative pathways to target the proteins for turnover in cell cycle regulation. First pathway allows for the selectivity of target molecule which is dependent on regulatory context in cell cycle. The second pathway activates the protein-ubiquitin ligase, which transfers ubiquitin to the target protein at particular points in cell cycle. Most commonly, the selective target destruction in cell cycle is achieved by a class of protein-ubiquitin ligase called SCF (Skp1/Cullin/F-box protein). The alternative form of protein-ubiquitin ligase activation is known as the anaphase-promoting complex/cyclosome (APC/C), which is activated through signaling pathways in cell cycle (Table 3, Figure 6).

B. The SCF

E3 ubiquitin ligases have been classified into three groups: the single and multi-subunit RING-finger type and the HECT-domain type. Most of the multi-subunit RING-finger type of E3 ligases contains a cullin protein (Cul1-5 and Cul7). The mammalian cullin-dependent ligase (CDL) is known as SCF (Skp1-Cul1-FBP) ligase (Figure 6). In this

ligase, the cullin subunit Cul1 interacts at the amino terminus with the crucial adaptor subunit Skp1 (S-phase-kinase-associated protein-1) and at the carboxyl terminus with a RING-finger protein (Rbx1) and a specific E2 enzyme or ubiquitin-conjugating enzyme (UBC), such as Ubc3-5. Skp1, in turn, binds to one of many FBPs (F-box binding protein). Each FBP has a number of specificities with substrates through a protein-protein interaction domain (Table 4).

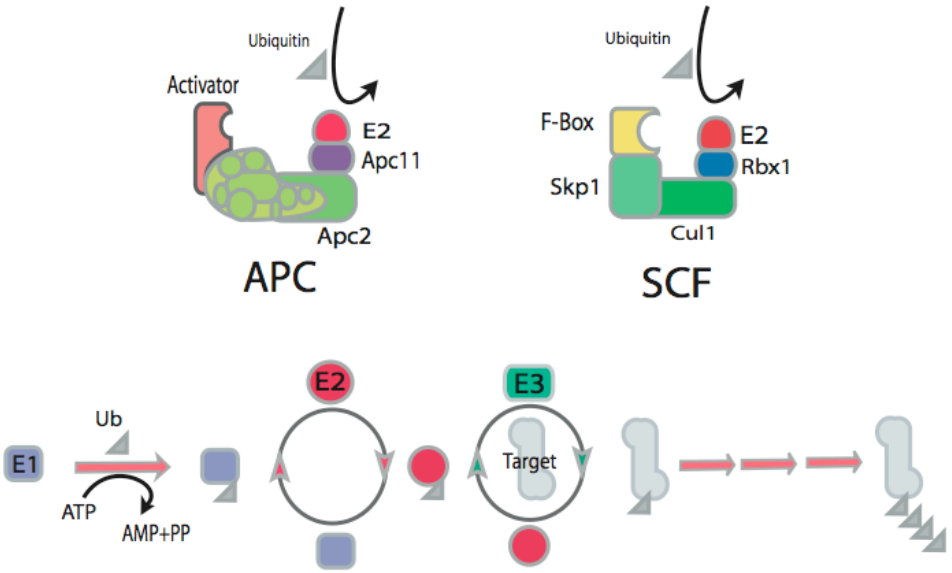

Figure 6. APC/SCF subunits and Ubiquitination pathway.

The APC is a large protein complex and consists of 11-13 subunits, including a RING subunit (Apc11) and a cullin (Apc2). Two activators (Cdc20 or Cdh1) are associated and required for APC activity. SCF contains three core subunits, including a RING protein Rbx1, a cullin Cul1, and Skp1. F-box protein interacts with Skp1 and positions the substrate for ubiquitination. Ubiquitin is covalently attached to ubiquitin activating enzyme (E1) by ATP dependent manner. E1 transfers the ubiquitin to ubiquitin conjugating enzyme (E2). Then, E2 associated with a target-specific ubiquitin ligase (E3) to catalyze the formation of peptidyl bond between ubiquitin and target protein.

The mammalian FBPs have been largely classified into three classes according to the structural class of their substrate-binding domains. FBWs ('FB' for F-box and 'W' for WD-40 repeat domain) have their substrate-binding domain with a β-propeller structure that is conserved in many protein-binding contexts and recognize specific Ser/Thr phosphorylation (pS/pT) consensus sequences : DpSGXXX(X)pS (where 'X' represents any amino acid) in Fbw1 (also known as β-Trcp1) ; and a variable L[I/L/P][pS/pT]P

sequence in Fbw7 and Cdc4. The domain of FBLs ('L' for Leucine-rich repeat (LRR)) is an arc-shaped α-β-repeat structure that is also found in many protein-binding contexts, including the extracellular binding domain of certain surface receptors. The third class of FBPs, the FBX does not contain WD-40 repeats or LRRs but often have different protein-protein interaction domains. (Cardozo and Pagano, 2004).

Table 3. The targets of ubiquitin-mediated proteolysis in cell cycle.

Ligase	*Regulator*	*Substrates*	*Function*
APC/C	Cdc20	Securin	Anaphase inhibitor
APC/C	Cdc20 / Cdh1	Cyclin B	Mitosis
APC/C	Cdc20 / Cdh 1	Cyclin A	S phase, mitosis
APC/C	Cdh1	Cdc20	Mitosis
APC/C	Cdh1	UbcH10, Cdh1	Mitosis
APC/C	Cdh1	Plk	Mitosis
APC/C	Cdh1	Aurora A	Mitosis
APC/C	Cdh1	Cdc6	DNA replication
APC/C	Cdh1	Geminin	Replication licensing
SCF	Cdc4	Cyclin E	G1-S
SCF	Cdc4	Cdc6	DNA replication
SCF	Skp2	$p27^{Kip1}$	G1-S transition CDK inhibitor
SCF	Skp2	$p21^{Cip1}$	G1-S transition CDK inhibitor
SCF	Skp2	Orc1	DNA replication
SCF	β-TrCP	Emi1	Mitosis APC/C inhibitor

C. APC/C

Eukaryotic cell cycle progression is driven by the living engine which is called Cyclin-dependent kinases (CDKs) (Murray, 2004). CDK activity is regulated accurately in spacious and timely dependent way in the cell and also cyclin itself is controlled by periodic accumulation and destruction. Mostly, the regulation of subsequent inactivation of cyclin-dependent kinase 1 by ubiquitin-proteolysis is very important event in mitosis (Peters, 2002). Cyclin A and B are destroyed through mitosis as substrates of the anaphase-promoting complex/Cyclosome (APC/C), a large multi-subunit E3 ubiquitin ligase (Harper et al., 2002). Similar with other E3 ligases, the APC/C has their own specific protein substrates and make an ubiquitin chains on them in order to indicate the signal to be destroyed by the 26S proteosome (Jackson et al., 2000). However, it still remains unclear how E3 enzymes with their targets are regulated and interacted with various accessory factors.

Table 4. Mammalian F-box proteins and their known functions.

	Mammalian F-box proteins (FBP)	Aliases	Human approved gene symbol	Main substrates	Comments
FBWs	Fbw1	β-Trcp1	*BTRC*	Emi1, Cdc25A, Wee1, β-catenin, IκB-family members	Gene knock-out phenotype : Defective spermatogenesis, subtle mitotic defects, centrosome overduplication.
	Fbw4	Dactylin	*SHFM3*	Unknown	Human split hand-out malformation (SHFM) gene.
	Fbw6		*FBXW6*	Unknown	In addition to being part of an SCF ligase, it also forms a complex with Skp1 and Cul7 ; the latter interacts with SV-40 large T antigen.
	Fbw7	Cdc4, Sel10	*FBXW7*	Cyclin E, Myc, Jun, Notch-1,4	Gene knock-out phenotype : Embryonic lethal at E11, probably due to morphogenetic cardiovascular defect. Mutations in ovarian and breast cancer cell lines.
FBLs	Fbl1	Skp2	*SKP2*	p21, p27, p57, p130	Gene knock-out phenotype : Hypoplasia in most organs, endoreplication, centrosome overduplication, defect of mitotic entry. Overexpressed in human tumors.
FBXs	Fbx1	Cyclin F	*CCNF*	Unknown	Function unknown, First-identified mammalian FBP.
	Fbx2		*FBXO2*	Unknown	Recognizes *N*-glycans. ER-associated degradation.
	Fbx5	Emi1	*FBXO5*	Unknown	Inhibitor of APC/C. Overexpressed in breast tumors.
	Fbx6		*FBXO6*	Unknown	Recognizes *N*-glycan.
	Fbx32	Mafbx, Atrogin1	*FBXO32*	Unknown	Involved in skeletal muscle atrophy. Higher expression in muscle cells.

Where APC/C, anaphase-promoting complex/Cyclosome ; Cdc, cell division cycle ; Cul7, cullin-7 ; E11, embryonic day 11 ; Emi1, early mitotic inhibitor 1 ; ER, endoplasmic reticulum ; IκB, inhibitor of nuclear factor (NF)κB ; FBL, F-box and leucine-rich-repeat protein ; FBW ; F-box and WD40-domain protein ; FBX, F-box-only protein ; SV40, simian virus-40 (Adapted from Cardozo and Pagano, 2004).

The APC/C is a multicomponent complex, composed of at least 10 subunits in mammals, including a Cullin homolog APC2 and a RING-H2 finger protein APC11 (Yu et al., 1998 ; Zachariae et al., 1998) (Figure 6). The APC shares homology with SCF

ubiquitin ligase in that contains a cullin and a RING-H2 finger protein as its catalytic core (reviewed in Jackson et al., 2000). The APC targets proteins containing a destruction box or a KEN box motif for ubiquitination-mediated proteolysis (Pfleger and Kirschner, 2000).

The APC regulates the timely destruction of mitotic regulators including cyclin A and B, the chromosome cohesion regulator securin, and many other mitotic regulatory kinases in order to direct progression through and exit from mitosis (Figure 7). The destruction of cyclin A early prometaphase is strongly linked with chromosome congression (den Elzen and Pines, 2001), destruction of securin is required for chromosome segregation and progression to anaphase (Cohen-Fix et al., 1996), and destruction of cyclin B is required for mitotic exit (Murray et al., 1989).

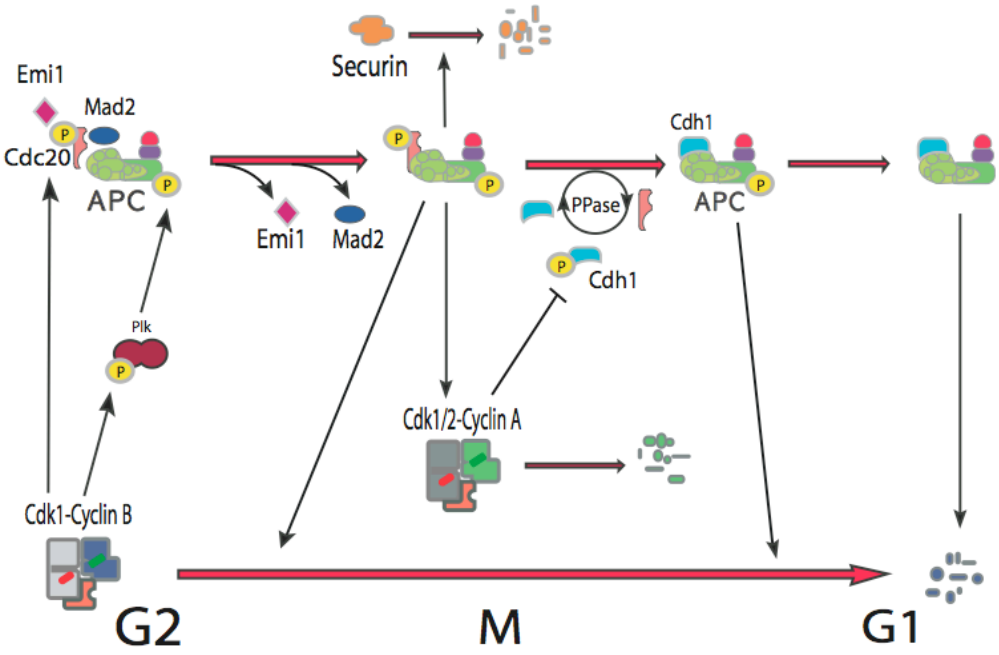

Figure 7. Regulation of APC activity.

APC^{Cdc20} is inhibited by mitotic spindle checkpoint, Mad2 and Emi1. In late prophase and early prometaphase, cyclin B-Cdk1 and Plk phosphorylate Emi1, triggering its destruction to activate APC^{Cdc20}. Mad2 is released from Cdc20 after all chromosomes attached to the mitotic spindle in metaphase and then APC^{Cdc20} degrades securin and cyclin B. Cdh1 is dephosphorylated through the phosphatase (PPase) Cdc14 and destruction of cyclin A at the end of mitosis, and able to bind to APC. APC^{Cdh1} ubiquitinates Cdc20 and inactivates APC^{Cdc20}. The activity of APC^{Cdh1} in turn degrades cyclin B and complete CDK inactivation until G1 (Adapted by Kotani et al., 1999).

1. APC/C Activators

The APC/C shows different substrate specificities depending on its association with the activating proteins Cdc20 and Cdh1 (Peters, 2006). Cdc20 binds and activates APC/C during mitosis, whereas Cdh1 activates APC/C in late mitosis and during G1 phase (Schwab et al., 1997; Visintin et al., 1997; Fang et al., 1998; Pines, 1999). The temporal order of APC activation by Cdc20 or Cdh1 is an important mechanism that prevents exit from mitosis before anaphase has occurred. Whereas the binding of Cdc20 to the APC depends on mitotic APC phosphorylation, Cdh1 can only bind to the APC once Cdh1 itself has been dephosphorylated by phosphatase Cdc14p (Zachariae et al., 1998; Kramer et al., 2000). However, the mechanism by which Cdc20 degradation is initiated and how cells switch from the APC^{Cdc20} to the APC^{Cdh1} form in late mitosis is not entirely clear.

Cdc20 activates the APC at the metaphase-to-anaphase transition to allow sister-chromatid segregation and to initiate the exit from mitosis (Fang et al., 1998) (Figure 8). As the cell reaches metaphase, mitotic CDKs activate the APC by phosphorylating core APC subunit which helps Cdc20 binding. APC^{Cdc20} then degrades target protein securin and the Mitotic cyclins, which allows inactivation of mitotic CDKs. APC^{Cdc20} is thought to be activated in late prophase or prometaphase, resulting in cyclin A destruction in early mitosis, whereas the destruction of securin and cyclin B is delayed until metaphase.

Cdc20 is replaced by another activator, Cdh1 in late mitosis. Cdh1 is phosphorylated and inactivated by cyclin A-CDKs (Lukas et al., 1999; Kramer et al., 2000; Sorensen et al., 2001). APC^{Cdh1} is not thought to be essential for mitotic progression but keeps continuing destruction of cyclins and CDK inactivation until the onset of S phase (Brandeis and Hunt, 1996). Moreover, APC^{Cdh1} also ubiquitinates other regulatory proteins which are not targeted by APC^{Cdc20}. Cdc20 is one of targets of APC^{Cdh1}. Interestingly, the APC^{Cdh1} autonomously degrades its own subunit UbcH10 (Ubiquitin-conjugating enzyme, E2) by ubiquitination-proteosome pathway in late G1. The APC/C substrates inhibit the autoubiquitinaiton of UbcH10, and the APC/C activity can be maintained as long as G1 substrates are present. Thus, cyclin A is stabilized and the re-accumulation of cyclin A inactivates APC/C by phosphorylating Cdh1 and promotes cell to enter S phase (Rape and Kirschner, 2004). Cyclin A is the only essential cyclin for S-phase entry in unperturbed cell cycles.

2. APC/C Inhibitors

The spindle checkpoint protein, Mad2 can bind to Cdc20 which is one of APC/C activators and has function on unattached kinetochores in prometaphase to inhibit the APC until chromosomes aligned in metaphase (Alexandru et al., 1999 ; Fang et al., 1998 ; Li et al., 1997 ; Wassmann and Benezra, 1998). One report showed that the inactivation of one Mad2 allele can markedly alter mitotic checkpoint function and result in premature anaphase and chromosome instability (CIN) in mammalian cells (Michel et al., 2001). Another spindle checkpoint protein, Bub1 can directly phosphorylates Cdc20 *in vitro* and this phosphorylation of Cdc20 by Bub1 inhibits ubiquitin ligase activity of APC^{Cdc20} catalytically (Tang et al., 2004). One single unattached kinetochore within a cell is enough

to activate spindle checkpoint, thus the spindle checkpoint might be partially responsible for inactivation of APC/C.

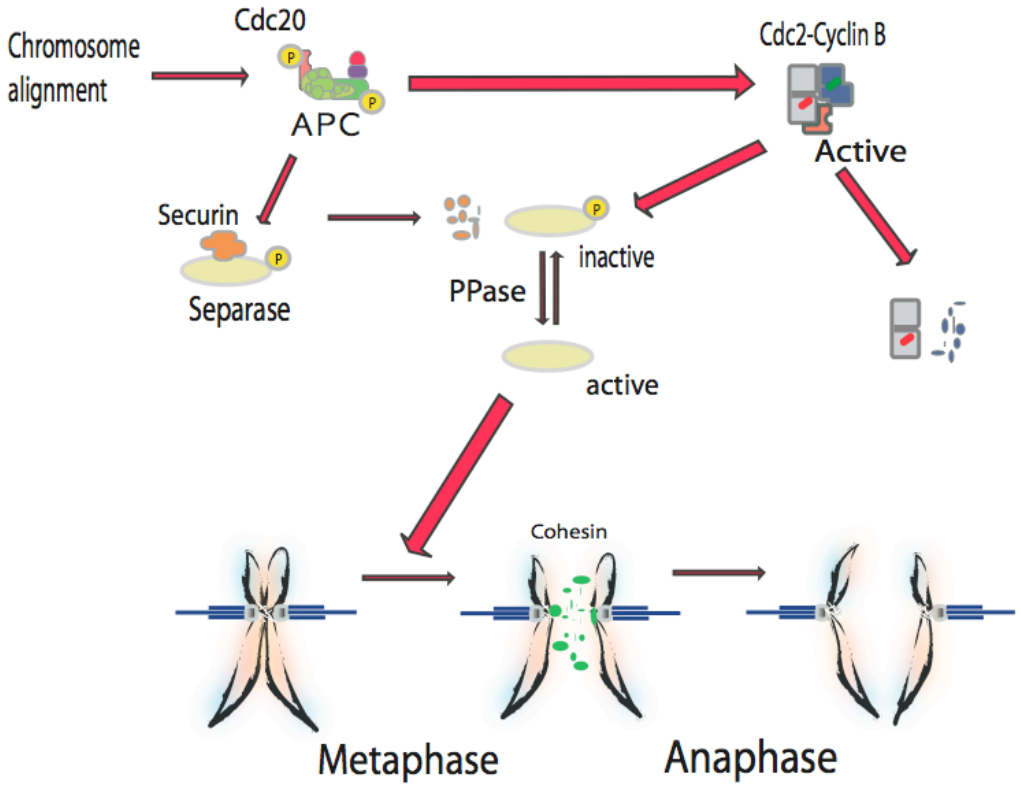

Figure 8. Regulation of the Metaphase-Anaphase transition and mitotic exit by APC^{Cdc20}.

Chromosomal alignment at metaphase plate constitutes the activation signal to APC^{Cdc20} for destruction of securin. Active separase cleaves the cohesin complexes, which are responsible for the maintenance of two sister-chromatids. APC^{Cdc20} also helps to activate separase through the degradation of cyclin B and other mitotic cyclins, which results in the removal of inhibitory phosphate residues from separase by protein phosphatase (PPase). The degradation of cyclin B induces Cdk1 inactivation, which is required for the mitotic exit (Adapted from Peters, 2002).

One of negative APC/C regulators and its homolog were identified and referred as Emi1 and 2 (Early mitotic inhibitor 1/2) (Reimann et al., 2001; Tung et al., 2004). Emi1 is accumulated in S phase and known to be degraded in early mitosis, independent of the APC activity, but dependent on CDK phosphorylation. The accumulation of Emi1 restrains the APC activity to inhibit the degradation of cyclin A and B during S/G2 phase. The predicted Emi1 protein is 392 residues long with an F-box, a zinc binding region (ZBR), five possible CDK phosphorylation sites, and two potential nuclear localization

sequences. Emi1 has homology to the regulator protein of *Drosophila* Cyclin A (Rca1) (Reimann et al., 2001). In *In vitro* assay, Emi1 ZBR directly binds to Cdc20 and inhibits the APC. Emi1 efficiently inhibits the APC^{Cdh1} *in vitro* (Reimann et al., 2001) and *in vivo* (Hsu et al. 2002). A conserved D-box in C-terminus and ZBR of Emi1 is required for association with APC through D-box receptor of Cdh1 or core APC/C to block substrate binding to the APC/C (Miller et al., 2006).

Emi1 destruction seems require for both phosphorylation on Ser/Thr-Pro sites by CyclinB/Cdk1 at the G2/M transition and $SCF^{\beta TrCP}$ ubiquitination in early mitosis through conserved DSGxxS motif, which is typically recognized by the β-TrCP protein. This degradation occurs just before the degradation of Cyclin A in prometaphase (Margottin-Goguet et al., 2003). The phosphorylation on DSGxxS motif in Emi1 is thought to involve in Plk1 whose the level oscillates in cell cycle, with a peak in mitotis (Moshe et al., 2004). In the absence of β-Trcp1, the stabilization of Emi1 in mitotic MEFs is observed (Guardavaccaro et al., 2003). The time faithful destruction of Emi1 for mitotic progression is important in genetic stability as a mitotic checkpoint and demonstrate possible role in tumor progression.

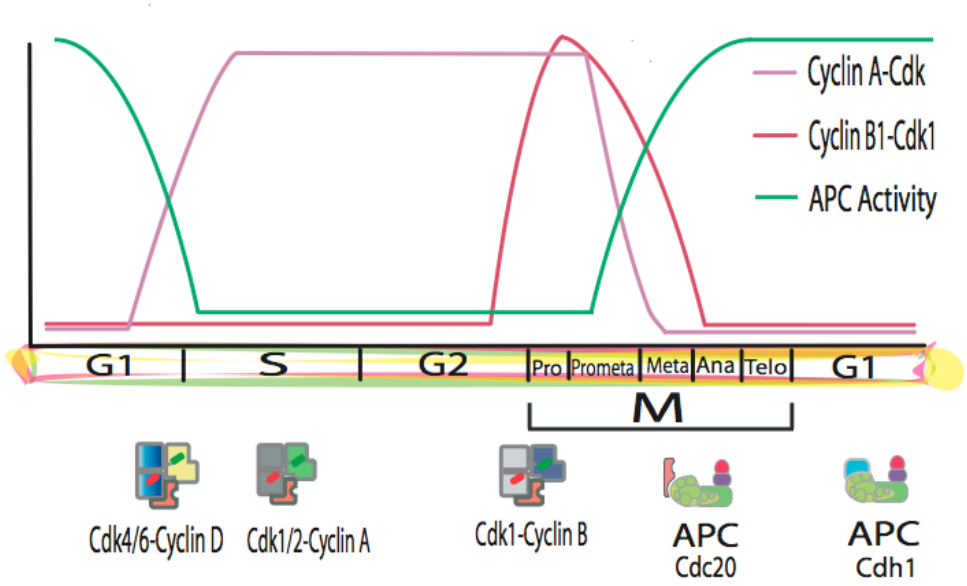

Figure 9. CDK and APC activities in cell cycle.

Emi1 overexpression blocks the mitotic entry by inhibiting the ubiquitination of Cyclin B *in vitro* (Reimann et al., 2001), and transient overexpression of Emi1 may result in centrosome overduplication, mitotic spindle abnormalities and failure of cytokinesis (Margottin-Goguet et al., 2003). Recent study showed that persistent Emi1 overexpression can lead to proliferation and induce genomic instability which causes tetraploidy (Lehman et al., 2006). In contrast, it has been recently proposed that Emi1 depletion can also create genomic catastrophe through DNA rereplication leading to

DNA damage checkpoint pathways (Machida and Dutta, 2007) and Emi1 is required to prevent the rereplication caused by uncoupled DNA replication with mitosis (Di Fiore and Pines, 2007).

Recent studies suggest that two different mechanisms are involved in Emi1 stabilization. The regulatory protein, Evi5 oncogene, has also been identified for the stabilization and the activity of Emi1. Evi5 contains Emi1 binding domain in N-terminus and a centrosomal-localization domain within its C-terminus. Evi5 seems to implicate the stabilization of Emi1 by co-localization of Evi5 at interphase centrosomes. Evi5 acts to stabilize Emi1 during interphase by blocking the ability of Polo-like kinases to trigger ubiquitin-dependent destruction of Emi1 by the $SCF^{\beta TrCP}$ complex (Eldridge et al., 2006). Another study identified Pin1 as another new regulator of Emi1 for its stabilization. Pin1 is a peptidyl-prolyl *cis/trans* isomerase, which isomerizes phosphorylated Ser/Thr-Pro peptide bonds (Lu et al., 1996). The isomerization catalyzed by Pin1 is involved in the conformation of the substrate, which affects cell functions through the regulation of enzymatic activity, protein stability or protein-protein interaction (Lu et al., 2002; Joseph et al., 2003; van Drogen et al., 2006). The study showed that Pin1 binds to Emi1 and Emi1 isomerization prevents its association with β-TrCP in an isomerization-dependent pathway (Bernis et al., 2006).

Purpose of thesis

In my thesis, we have investigated that DNA damage-induced inactivation of APC is dependent on p21-mediated Emi1 degradation. It has been reported that the Emi1 degradation is essential for activating APC in early mitosis to destroy cyclins, which results in maintaining CDK inactivation during mitosis and until early G1. Low CDK activity is also required for pre-RC formation to replicate DNA in late mitosis. This precise regulation of activities between CDK and APC is essential for cell to couple DNA synthesis with mitosis in each cell cycle (Figure 9). However, in absence of p21, Emi1 is stabilized and inactivates the APC/C after DNA damage. In addition, high CDK activity keeps APC inactive during mitosis and induces DNA replication after a failure of mitosis. We also find that this Emi1 stabilization is regulated by Rb-E2F pathway as well as CDK activity after DNA damage. Furthermore, DNA replication after DNA damage occurs concomitantly with the stabilization of Emi1 and cyclins, implying that the APC/C is needed to be inactivated in S phase for DNA replication with failed mitosis.

RESULTATS & CONCLUSION

PREMIERE PARTIE : Manuscrit pour la publication

DNA damage triggers p21^{WAF1}dependent Emi1 degradation and activation of Anaphase Promoting Complex (APC) to promote cell cycle arrest

MANUSCRIPT

DNA damage triggers p21^{WAF1} dependent Emi1 degradation and activation of Anaphase Promoting Complex (APC) to promote cell cycle arrest

Running title: DNA damage induced APC activation

ABSTRACT

We have analyzed the role of $p21^{WAF1}$ in G2-M phase checkpoint control and the generation of polyploidy after DNA damage. It has been shown that following DNA damage, $p21^{+/+}$ cells arrest with 4N DNA content whereas $p21^{-/-}$ cells display a ploidy of 8N. We find that DNA damage activates the Anaphase Promoting Complex (APC) in $p21^{+/+}$ cells but not in $p21^{-/-}$ cells. This p21 dependent APC activation after DNA damage is linked to the inhibition of CDK activity. Moreover, p21 dependent proteolysis of Emi1, a negative regulator of APC, and retinoblastoma protein (Rb) mediated transcriptional repression of Emi1 contribute to APC activation after irradiation. Rb down-regulation in irradiated $p21^{+/+}$ cells by siRNA resulted in Emi1 mRNA and protein expression, APC inactivation and in the accumulation of cells with 8N DNA content. Finally, caffeine or Chk1 siRNA overcomes the irradiation induced 4N arrest and generates cells with 2N DNA content during a window of opportunity most likely defined by $p21^{WAF1}$ dependent APC activation. Taken together our results show how DNA damage induced 4N arrest is held in place by $p21^{WAF1}$.

Cell cycle checkpoints safeguard genome integrity. p53 tumor suppressor has an important role in checkpoint response to DNA damage. After DNA damage, p53 activates the transcription of several genes including $p21^{WAF1}$, an inhibitor of cyclin dependent kinases (CDKs)[1]. G1 arrest after DNA damage is mediated in part by p53 dependent increase in transcription of $p21^{WAF1}$ and the inhibition of the Cdk-cyclin activity[2-6].

After DNA damage, cells arrest at the G1 phase with 2N DNA content or at the G2 phase with 4N DNA content[7]. A role of $p21^{WAF1}$ in G2 arrest was suggested by studies in which the ectopic expression of p53 or $p21^{WAF1}$ (here-after referred to as p21) led to an arrest in both the G1 and G2 phases of the cell cycle[8-11]. The importance of p21 in the G2 DNA damage response became evident when diploid human colorectal cell line HCT116 lacking p21 failed to sustain G2 arrest after γ radiation[12,13]. HCT116 cells lacking p21 continue to cycle and attain a DNA content of 8N[12]. An increase in cells with a DNA content greater than 4N has also been observed in γ irradiated $Rb^{-/-}$ fibroblasts[11].

The Anaphase promoting complex (APC) is a multi-protein complex with E3-ubiquitin ligase activity[14-16]. Ubiquitination of specific substrates by APC targets them for degradation by the proteasome. APC is present throughout the cell cycle, however, its activity is high only from late mitosis until late G1[14,15]. APC activity regulates progression through mitosis to G1 and its substrates in mammalian cells include the inhibitor of anaphase onset (securin), cyclins (A2 and B1), other mitotic kinases (polo-like kinase 1, aurora kinases) and regulators of pre-replication complex formation (Cdc6, Geminin)[14-24]. APC is activated upon association with Cdc20 (also known as Slp1, fizzy, p55CDC)[14,15] or with Cdh1 (also known as Hct1, Ste9, Srw1, fizzy related)[15]. In mammalian cells, the binding of Emi1 (*early mitotic inhibitor 1*) to Cdc20 and Cdh1 inhibits the ubiquitination activity of active APC^{Cdc20} and APC^{Cdh1} (ref. 26). In human cells, Emi1 protein levels are high in G1-S, S phase

and early mitosis. Emi1 restrains the activation of APC in G1-S, S phase and in late G2[25]. Emi1 destruction in prophase by $SCF^{\beta TrCP}$ ubiquitin ligase is important for the activation of APC and the entry of cells into mitosis[26,27].

In this study, we have examined the mechanism underlying p21 dependent arrest with 4N DNA content after DNA damage. We show that p21 dependent proteolysis of Emi1 and Rb mediated suppression of Emi1 expression contribute to APC activation after DNA damage. After irradiation, Chk1 inhibition overcomes the 4N arrest and generates cells with 2N DNA. APC activation then functions as a point of no return for cells arrested with 4N DNA content. The irradiation induced generation of 8N $p21^{-/-}$ cells is, however, not abolished by Chk1 inhibition.

RESULTS

DNA damage activates APC in a p21 dependent manner

To study the role of p21 in the cellular response to DNA damage, parental HCT116 containing wild type p21 ($p21^{+/+}$) and HCT116 cells in which p21 gene was disrupted were used[12,13]. Asynchronous $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells were γ irradiated and treated with nocodazole, a microtubule disrupting agent, as described previously[13,28]. By 15 hours after γ irradiation, most of the cells progressed to G2 phase as indicated by the accumulation of cells with a 4N DNA content as shown previously[13,28] (**see Supplementary Information, Fig. S1a**). Irradiation of $p21^{+/+}$ cells resulted in an increase in p21 protein (**Fig. 1a**). We noticed that cyclin B1 protein disappeared in γ irradiated $p21^{+/+}$ cells whereas its levels remained relatively unchanged after irradiation of $p21^{-/-}$ cells **(Fig. 1a)**. Cyclin B1 protein is degraded by APC[15-17], we therefore tested the stability of other known substrates of APC after irradiation such as Cdc20 and Aurora A. Protein levels of both Cdc20 and Aurora A decreased in $p21^{+/+}$ cells after irradiation whereas their levels remained high in irradiated $p21^{-/-}$ cells **(Fig. 1a)**. A decrease in APC substrates was also observed upon irradiation of asynchronous IMR90, a non-transformed human fibroblast cell line (**data not shown**).

The most likely explanation of our results was that APC was activated in irradiated $p21^{+/+}$ HCT116 cells but not in $p21^{-/-}$ HCT116 cells after irradiation. APC activity was therefore tested using an *in vitro* APC-dependent ubiquitination assay. APC^{Cdc20} was isolated by immunoprecipitation with Cdc20 specific antibody and APC^{Cdc20} activity was determined using cyclin B1 as a substrate[29]. Polyubiquitination of cyclin B1 was clearly observed in extracts from γ irradiated $p21^{+/+}$ cells indicating that APC^{Cdc20} was activated after irradiation **(Fig. 1b, upper panel)**. APC^{Cdh1} was immunoprecipitated with Cdh1 specific antibody and

APC^{Cdh1} activity was determined using Cdc20 as a substrate (**Fig. 1b, lower panel**). Activation of both APC^{Cdc20} and APC^{Cdh1} occurred in γ irradiated $p21^{+/+}$ cells. APC^{Cdc20} activity declined at later times after γ irradiation probably due to degradation of Cdc20 by APC^{Cdh1} (ref. 32)(**Fig. 1a**). In sharp contrast to $p21^{+/+}$ cells, activation of APC^{Cdc20} and APC^{Cdh1} was not observed in $p21^{-/-}$ cells after γ irradiation. There was thus a good correspondence between APC activation and the observed stability of APC substrates after DNA damage.

APC subunit Cdc27, also known as APC3, undergoes phosphorylation mediated electrophoretic mobility shift which coincides with APC activation[15,29]. The supershifted form of Cdc27 was readily observed in extracts from both $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells at 24 hours after irradiation (**Fig. 1c**).

The effect of p21 on γ irradiation induced degradation of APC substrates was further examined by synchronizing cells by a double thymidine block or by treatment with hydroxyurea (HU). At 2.5 h after release into fresh medium, 60-80% of the cells were BrdU positive and there was no MPM2 signal (**data not shown and see Supplementary Information, Figs. S1a, c**). Cells released from HU (**see Supplementary Information, Fig. S1d)** or from double thymidine block continue to cycle normally (**data not shown**). Cells released from the respective synchronization regimes for 2.5 h were irradiated and tested for the degradation of APC substrates **(Figs. 1d, e)**. Similar to the results obtained with γ irradiation of asynchronized cells treated with nocodazole, protein levels of a panel of APC substrates decreased in synchronized $p21^{+/+}$ but not in $p21^{-/-}$ HCT116 cells after irradiation **(Figs. 1d, e)**. Histone H2AX phosphorylation, indicative of double strand breaks, was easily observed after DNA damage **(Figs. 1d, e)**. In all subsequent experiments, the cells were synchronized with HU, released for 2.5 h and then γ irradiated unless otherwise specified.

A failure of $p21^{-/-}$ cells to degrade APC substrates was likewise observed after

treatment of HU synchronized cells with adriamycin **(Fig. 1f)**. Adriamycin intercalates with DNA and stabilizes topoisomerase II-DNA cleavable complexes thus generating double strand breaks.

Inhibition of CDK activity activates APC in γ irradiated $p21^{-/-}$ cells

p21 inhibits cell cycle progression by suppressing CDK activity. p21 protein increased in $p21^{+/+}$ HCT116 cells by 15 h after DNA damage (**Fig. 1**). When synchronized $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells were γ irradiated, Cdk2 and Cdk1 associated kinase activities were both low in irradiated $p21^{+/+}$ cells but elevated in $p21^{-/-}$ cells **(Fig. 2a)** as shown previously[13,28,30]. Consistent with the low kinase activity in $p21^{+/+}$ cells, there was an increased association of p21 with Cdk2 and Cdk1 after γ irradiation **(Fig. 2b)**. Cdk2 and Cdk1 associated activities were also low in $p21^{+/+}$ cells but not in $p21^{-/-}$ cells after adriamycin treatment (**data not shown**).

We considered the possibility that the high CDK activity in $p21^{-/-}$ HCT116 cells may be linked to the failure to activate APC. Synchronized $p21^{-/-}$ cells were either γ irradiated or treated with adriamycin. The cells were treated with roscovitine, a reversible inhibitor of Cdk1 and Cdk2 associated kinase activities[31]. Indeed, the addition of roscovitine to γ irradiated **(Fig. 2c, upper panel)** or adriamycin treated **(Fig. 2e)** $p21^{-/-}$ cells resulted in the degradation of APC substrates. Erk1 and Erk2, are sensitive to roscovitine but at 20 to 50 fold higher concentrations than Cdks[31]. Nevertheless, we confirmed that APC substrate degradation in roscovitine treated $p21^{-/-}$ cells was not due to Erk1 or Erk2 inhibition. First, Erk1/Erk2 phosphorylation remained unchanged upon roscovitine treatment of irradiated $p21^{-/-}$ cells **(Fig. 2c, lower panel)**. Furthermore, treatment of γ irradiated $p21^{-/-}$ cells with UO126, an inhibitor of Erk1/Erk2 activation, resulted in the inhibition of Erk1/Erk2 phosphorylation but it did not lead to the degradation of APC substrates in irradiated cells **(Fig. 2d)**. Our

results are thus consistent with a model in which the inhibition of CDK activity after DNA damage leads to APC activation in $p21^{+/+}$ cells.

CDK inhibition prevents both DNA synthesis and the increase in ploidy of $p21^{-/-}$ cells after DNA damage

One outcome of DNA damage induced by γ irradiation or adriamycin in $p21^{-/-}$ HCT116 cells is that the cells become polyploid, exhibiting a DNA content of $8N^{12}$. After irradiation, both $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells progressed to 4N **(Fig. 3a)**. Afterwards, irradiated $p21^{+/+}$ cells remained arrested with a DNA content of 4N at longer times **(Figs. 3a, c)**. In contrast to $p21^{+/+}$ cells, similarly γ irradiated $p21^{-/-}$ cells underwent DNA synthesis 24 h after irradiation **(Fig. 3b)**. Subsequently the γ irradiated $p21^{-/-}$ cells showed a progressive decrease in cells with 4N DNA content and an increase in population with 8N DNA content **(Figs. 3a, c)** consistent with the DNA synthesis data shown in **Figure 3b**. The significance of APC activation in maintaining the DNA ploidy after DNA damage was tested by the activation of APC in $p21^{-/-}$ cells through the down regulation of CDK activity by roscovitine. Indeed, treatment of γ irradiated $p21^{-/-}$ cells with roscovitine completely abolished DNA synthesis (**see Fig. 3d legend**) and prevented the generation of cells with 8N DNA **(Figs. 3d, e)**.

Adriamycin treated $p21^{-/-}$ cells also exhibit a DNA content of 8N whereas similarly treated $p21^{+/+}$ cells arrest in 4N **(Fig. 3f)**. Treatment of $p21^{-/-}$ cells with roscovitine prevented the generation of cells with 8N DNA after adriamycin induced DNA damage **(Fig. 3f)**.

DNA damage induced arrest of $p21^{+/+}$ cells with 4N DNA content is maintained in part by Rb through regulation of APC activity

Rb deficient fibroblasts are impaired in their ability to arrest in G1 after DNA

damage[11,32,33]. The DNA damage induced G1 checkpoint induced by p21 is mediated in part by Rb[32,33]. p21 dependent CDK inhibition after γ irradiation leads to the accumulation of hypophosphorylated Rb. Indeed, hypophosphorylated Rb accumulated in γ irradiated $p21^{+/+}$ cells but not in $p21^{-/-}$ cells (**Fig. 1**). We tested whether the down-regulation of Rb in $p21^{+/+}$ HCT116 cells by siRNA would generate cells with 8N DNA content after DNA damage accompanied by APC activation (**Fig. 4a**). Both control and Rb siRNA transfected cells $p21^{+/+}$ cells progressed to 4N after irradiation (**Fig. 4a**). Afterwards, as expected, γ irradiated control siRNA transfected cells arrested with 4N DNA content. Irradiated Rb siRNA transfected $p21^{+/+}$ cells, however, incorporated BrdU (**Fig. 4b**) and the cells accumulated with 8N DNA content (**Fig. 4a, c**). Interestingly, APC substrate degradation progressed similarly in both irradiated Rb siRNA and control siRNA transfected cells until 24 h (**Fig. 4d**). Subsequently, the APC substrates accumulated in irradiated Rb siRNA transfected $p21^{+/+}$ cells whereas the APC substrates were almost completely degraded in irradiated control siRNA transfected $p21^{+/+}$ cells. Notably, p21 protein levels were increased after irradiation of both control siRNA and Rb siRNA transfected $p21^{+/+}$ cells (**Fig. 4d**). These results show that p21 dependent arrest with 4N DNA content after DNA damage is mediated in part by Rb through regulation of APC function. The kinetics of APC activation in γ irradiated $p21^{+/+}$ cells in which Rb is down-regulated further shows that Rb functions in p21 dependent 4N arrest by maintaining APC activity and not in initiating APC activation.

Emi1 down regulation after DNA damage: proteolysis of Emi1 protein and repression of Emi1 mRNA

Emi1 is a negative regulator of APC activity[34,35]. We tested whether Emi1 protein levels were regulated after DNA damage. Synchronized $p21^{+/+}$ and $p21^{-/-}$ cells were γ irradiated or treated with adriamycin and the levels of Emi1 protein were tested (**Fig. 5a**).

Interestingly, Emi1 protein levels decreased in $p21^{+/+}$ cells after DNA damage and Emi1 was undetectable at longer times after DNA damage. In contrast, Emi1 protein levels remain relatively high even at longer times after DNA damage in $p21^{-/-}$ cells.

The decrease of Emi1 protein after DNA damage in $p21^{+/+}$ HCT116 cells could be completely rescued by the addition of proteasome inhibitor LLnL at 15 h and to a lesser extent if LLnL was added later at 24 h (**Fig. 5b**). The half-life of Emi1 protein was also decreased at 15 h after irradiation (**Fig. 5c**). The decrease in Emi1 protein in $p21^{+/+}$ cells in response to DNA damage is thus proteasome dependent. F-box protein β-Trcp1 has been shown to regulate the destruction of Emi1 protein[26,27]. Cdk1-cyclin B stimulates Plk1 dependent phosphorylation of serines located in the DSGxxS motif in Emi1 leading to *in vitro* degradation of Emi1 by $SCF^{\beta\text{-Trcp1}}$ (ref. 38, 39). We tested whether the failure of Emi1 protein degradation in $p21^{-/-}$ cells after DNA damage could be explained by the lack of Plk1 activity these cells. Plk1 activity was high in $p21^{-/-}$ cells as early as 9 h after γ irradiation, quite similar to $p21^{+/+}$ cells (**Fig. 5d**). Thus, although both Plk1 and Cdk1 activities (**Fig. 2a**) are high in irradiated $p21^{-/-}$ cells, Emi1 protein was not degraded. Intriguingly, Emi1 protein levels were low in cells in which CDK activity was inhibited (irradiated $p21^{+/+}$ cells) and Emi1 was stable in cells with the high CDK activity (irradiated $p21^{-/-}$ cells) (**Fig. 5a**). Consistent with this observation, Emi1 protein was rapidly degraded by roscovitine treatment of irradiated $p21^{-/-}$ cells (**Fig. 5e**). Emi1 protein levels remain unchanged upon treatment of irradiated $p21^{-/-}$ cells with UO126 (**see Supplementary information, Fig. S2**). Finally, Emi1 protein levels were reduced quickly, within 2-4 hours, after treatment of asynchronous cells with roscovitine (**Fig. 5f**). Together these results suggest an alternative pathway targeting Emi1 protein degradation in conditions where CDK activity is low such as after DNA damage.

We tested whether a reduction in Emi1 mRNA levels contributes to the prominent

decrease of Emi1 protein in γ irradiated $p21^{+/+}$ HCT116 cells which cannot be completely rescued by LLnL at later times after DNA damage (**Fig. 5b**). Real-time PCR analysis revealed a striking reduction in Emi1 mRNA levels in γ irradiated $p21^{+/+}$ cells (**Fig. 5g**). The situation was different in $p21^{-/-}$ cells in which the Emi1 mRNA levels remain relatively unchanged after irradiation (**Fig. 5g**). Moreover, Emi1 mRNA was efficiently reduced in irradiated $p21^{-/-}$ cells treated with roscovitine (**Fig. 5h**).

As Rb-E2F has been proposed to regulate Emi1 transcription[25], we analyzed the Emi1 mRNA levels in γ irradiated Rb siRNA transfected $p21^{+/+}$ cells. Real-time PCR analysis revealed that Emi1 mRNA was reduced to 35% at 15 h and further to 17% at 48 h in irradiated control siRNA transfected $p21^{+/+}$ cells (**Fig. 5i**). The situation was different in irradiated Rb siRNA transfected $p21^{+/+}$ cells. Emi1 mRNA decreased to 32% at 15 h and then it increased to 54% at 48 h after irradiation (**Fig. 5i**). The Emi1 protein levels were reduced in both control and Rb siRNA transfected $p21^{+/+}$ cells by 24 h. By 48 h, however, there was an increase in Emi1 protein in Rb siRNA treated $p21^{+/+}$ cells that was co-incident with the accumulation of APC substrates (**Fig. 4d**) and preceded the incorporation of BrdU and the generation of 8N cells (**Figs. 4b, c**). These results suggest that DNA damage induced transcriptional repression of Emi1 in $p21^{+/+}$ cells is mediated in part by Rb. Taken together our results show that p21 acts at two levels, proteolysis of existing Emi1 protein and Rb mediated repression of Emi1 mRNA there-by maintaining APC in its active state.

We next tested whether the down-regulation of Emi1 in irradiated $p21^{-/-}$ cells by siRNA would prevent the generation of cells with 8N DNA content. Emi1 siRNA transfected $p21^{-/-}$ cells released from HU block and irradiated, progressed to 4N similarly to control siRNA treated cells (**Fig. 5k**). Both DNA synthesis **(Fig. 5l)** and the generation of cells with 8N DNA content **(Fig. 5k)** was substantially reduced in γ irradiated Emi1 siRNA transfected $p21^{-/-}$ cells as compared to irradiated control siRNA transfected cells. Emi1 siRNA mediated

down-regulation of Emi1 was verified by immunoblotting **(Fig. 5m)**. These results show that DNA damage induced generation of cells with 8N DNA content fails to occur in cells with down regulated Emi1.

Differential roles of ATR/ATM/Chk1 kinases and p21 in regulating DNA damage induced 4N arrest

DNA damaging agents such as ionizing radiation and UV mediate checkpoint response by activating Ataxia-telangiectasia mutated (ATM) and/or ataxia-telangiectasia and Rad3-related (ATR) protein kinases[36,37]. ATM and ATR are critical early mediators of the DNA damage response and they phosphorylate and activate Chk1 and/or Chk2, two structurally unrelated serine/threonine kinases with overlapping substrate specificities. To investigate whether ATR/ATM pathway plays a role in DNA damage induced 4N arrest, $p21^{+/+}$ HCT116 cells were treated with caffeine (**Fig. 6a**) or with UCN-01 (**Figs. 6b, c**) at 6 or 15 h after irradiation. Caffeine is an inhibitor of ATR and ATM kinases[38] whereas UCN-01 inhibits Chk1[39] and Chk2 kinases[40]. While control cells arrested with 4N DNA content, caffeine or UCN-01 treatment resulted in the generation of cells with 2N DNA content. Similar results were obtained when $p21^{+/+}$ cells synchronized by double thymidine block were treated with UCN-01 at 6 h after irradiation (**see Supplementary Information, Fig. S3**). These results show that caffeine or UCN-01 overcome the 4N arrest of γ irradiated $p21^{+/+}$ cells. The cells go through mitosis, as verified by MPM2 positivity, to generate cells with 2N DNA content (**Fig. 6d**). We also observed that APC substrates were degraded earlier in caffeine (**see Supplementary Information, Fig. S4**) or UCN-01 treated cells (**data not shown**). Once in 2N the $p21^{+/+}$ cells, however, arrest and do not cycle (**Fig. 6e**).

We show above that the addition of caffeine (**Fig. 6a**) or UCN-01 (**Fig. 6c**) at 15 h after γ irradiation overcame the 4N arrest of irradiated $p21^{+/+}$ cells and generated cells with

2N DNA content. The addition of UCN-01 (**Fig. 6f**) or caffeine (**data not shown**) at 24 h after γ irradiation, however, could not overcome the 4N arrest of irradiated $p21^{+/+}$ cells to generate cells with 2N DNA content. There is thus a window of opportunity after irradiation during which caffeine or UCN-01 can overcome the 4N arrest and generate cells with 2N DNA content.

Next, we tested whether the effect of caffeine and UCN-01 in overcoming the 4N arrest of irradiated $p21^{+/+}$ cells is mediated by inhibiting Chk1. Chk1 was down-regulated by transfecting $p21^{+/+}$ cells with Chk1 siRNA (**Fig. 6g**). Both control and Chk1 siRNA transfected cells $p21^{+/+}$ cells progressed to 4N after irradiation (**Fig. 6g**). Afterwards, $p21^{+/+}$ cells in which Chk1 is down regulated proceed from 4N to generate cells with 2N DNA content, whereas control siRNA treated cells remain arrested in 4N. Degradation of cyclin B1 was accelerated in irradiated Chk1 siRNA treated cells as compared to control siRNA treated cells (**Fig. 6g**). These results show that DNA damage induced 4N arrest is Chk1 dependent.

We then tested the effect of UCN-01 and caffeine on the DNA damage response of γ irradiated $p21^{-/-}$ HCT116 cells, especially their failure to arrest in 4N and to generate cells with 8N DNA content. $p21^{-/-}$ cells were treated with caffeine (**Fig. 6h**) or with UCN-01 (**data not shown**) at 6 h or 15 h after irradiation. Interestingly, neither caffeine nor UCN-01 treatment of γ irradiated $p21^{-/-}$ cells prevented the generation of 8N cells. Consistent with these results, down regulation of Chk1 in $p21^{-/-}$ cells by siRNA failed to abolish the generation of cells with 8N DNA content (**data not shown**). Together these results show that the DNA damage induced 4N arrest is abolished by Chk1 inhibition whereas the generation of 8N cells is not.

DISCUSSION

Irradiated $p21^{+/+}$ cells have been shown to arrest with 4N DNA content whereas cells lacking p21 become polyploid, exhibiting a DNA content of 8N[12]. We show here that the p21 dependent arrest with 4N DNA content after DNA damage is mediated in part by regulation of APC function. DNA damage leads to the activation of both APC^{Cdc20} and APC^{Cdh1} in $p21^{+/+}$ cells but not in $p21^{-/-}$ cells. The activation of APC after irradiation is brought about by p21 dependent proteolysis of Emi1 and in part by Rb mediated repression of Emi1 transcript levels. In γ irradiated $p21^{+/+}$ cells, CDK activity is inhibited, Rb is hypo-phosphorylated, Emi1 protein is degraded and Emi1 mRNA is repressed. siRNA mediated down-regulation of Rb in $p21^{+/+}$ cells leads to the expression of both Emi1 mRNA and protein after irradiation resulting in the inactivation of APC. In contrast to irradiated $p21^{+/+}$ cells, CDK activity is elevated, Rb remains hyper-phosphorylated and Emi1 protein and mRNA are high in γ irradiated $p21^{-/-}$ cells. Treatment of γ irradiated $p21^{-/-}$ cells with CDK inhibitor, roscovitine, triggers Emi1 protein degradation, represses Emi1 mRNA and activates the APC. This results in the arrest of irradiated $p21^{-/-}$ cells with 4N DNA content. APC activation after irradiation brought about by p21 dependent proteolysis of Emi1 and in part by Rb mediated transcriptional repression of Emi1 thus plays an important role in 4N arrest. Taken together our results reveal how APC activation can occur after DNA damage and suggest a physiological role for APC activation in DNA damage checkpoint induced 4N arrest in mammalian cells.

In support of the regulation of APC activity by p21, we show that APC activation after DNA damage is dependent on the inhibition of CDK activity. CDK activity is inhibited upon DNA damage in $p21^{+/+}$ cells whereas CDK activity is high in $p21^{-/-}$ cells. As DNA

damage leads to the activation of APC in $p21^{+/+}$ cells but not in $p21^{-/-}$ cells, it is interesting that treatment of γ irradiated $p21^{-/-}$ cells with CDK inhibitor, roscovitine, leads to APC activation. Further, we find that Emi1 protein stability is regulated as a function of CDK activity. Emi1 protein levels decrease dramatically at 15 h in γ irradiated $p21^{+/+}$ cells but not $p21^{-/-}$ cells. However, roscovitine treatment of irradiated $p21^{-/-}$ cells results in the reduction of Emi1 protein. Finally, treatment of asynchronous cells with roscovitine results in the reduction of Emi1 protein within a few hours, while the levels of other proteins remain relatively unchanged.

The decrease in Emi1 protein levels at 15 h in irradiated $p21^{+/+}$ cells can be prevented by proteasome inhibition. The degradation of Emi1 reported here differs from the destruction of Emi1 by F box protein β-Trcp1, recently shown to be regulated by Cdk1-cyclin B and Plk1[41,42]. For instance, we find that although both the CDK and the Plk1 activities are high following DNA damage of $p21^{-/-}$ cells, yet Emi1 protein is elevated. Additionally, roscovitine leads to Emi1 degradation in $p21^{-/-}$ cells after DNA damage. Together our results suggest that under certain conditions of low CDK activity, there may be an alternative pathway to degrade Emi1 protein perhaps involving another ubiquitin ligase. It is equally possible that low CDK activity may result in the degradation of an Emi1 associated protein, leading to Emi1 destabilization.

Our results suggest that the inhibition of CDK activity by p21 after DNA damage acts at two levels, proteolysis of existing Emi1 protein as discussed above and the repression of Emi1 mRNA. Emi1 mRNA levels are dramatically reduced in γ irradiated $p21^{+/+}$ cells, consistent with the absence of Emi1 protein at later times after DNA damage. Although Emi1 mRNA levels remain relatively unchanged after irradiation of $p21^{-/-}$ cells, CDK inhibition by roscovitine, however, leads to the reduction of Emi1 mRNA. Furthermore, in irradiated Rb siRNA transfected $p21^{+/+}$ cells, Emi1 mRNA increased at later time after

irradiation whereas Emi1 mRNA levels were reduced in control siRNA transfected $p21^{+/+}$ cells.

Our findings that APC activation in $p21^{+/+}$ cells after DNA damage plays an essential role in 4N arrest offers new insight into the mechanism of cell cycle arrest after DNA damage. The arrest of $p21^{+/+}$ cells with 4N DNA content shares common features with γ irradiation induced G1 arrest[6]. In both cases, the p21 protein levels are high, Rb is hypo-phosphorylated and CDK activity is inhibited. The DNA damage induced G1 checkpoint by p21 is mediated in part by Rb[32,33]. We show here that the DNA damage induced p21 dependent arrest in 4N is likewise mediated in part by Rb, and furthermore, the suppression of Emi1 mRNA appears to play an important role. The loss of Rb function in $p21^{+/+}$ cells ultimately results in Emi1 mRNA synthesis, APC inhibition and in a failure of cells to remain arrested with 4N DNA content. The initial activation of APC in γ irradiated $p21^{+/+}$ cells in which Rb is down regulated further shows that the p21 increase after irradiation and the resulting degradation of Emi1 protein activates APC, and that Rb most likely functions in p21 dependent 4N arrest by maintaining APC activity and not in initiating APC activation.

Normal cell division is critical for maintaining genome ploidy. It has been suggested that $p21^{-/-}$ HCT116 cells become polyploid after DNA damage because the cells enter mitosis, as evidenced by MPM2 positivity, but fail to execute mitosis properly[13,28]. The cells exit mitosis and enter interphase with 4N DNA content as a result of improper mitosis rather than 2N DNA content expected of cells that have executed mitosis properly. The 4N $p21^{-/-}$ cells then undergo a new round of DNA synthesis after exiting from mitosis and become 8N. Indeed we find that irradiated 4N $p21^{-/-}$ cells undergo DNA synthesis. Our finding that APC is not activated in irradiated $p21^{-/-}$ cells may contribute to the failure of these cells to execute mitosis properly.

We find that caffeine, UCN-01 and Chk1 siRNA overcome the 4N arrest of irradiated

$p21^{+/+}$ cells and the cells proceed through mitosis and generate a 2N population. The generation of a 2N population demonstrates that the cells had properly exited mitosis and divided. As the majority of the cells become 2N, these results further show that in the presence of UCN-01 or caffeine, DNA damage does not interfere with chromosome segregation or cytokinesis. While the mechanism preventing the generation of 2N cells from 4N cells after irradiation is abolished by Chk1 inhibition, the generation of cells with 8N DNA content is not abolished by Chk1 inhibition.

We observed that caffeine and UCN-01 overcome the 4N arrest of irradiated $p21^{+/+}$ cells when added to cells at 15 h after irradiation. However, treatment of cells with caffeine or UCN-01 at 24 h after irradiation did not abolish the 4N arrest. These results show that there is a window of opportunity after irradiation during which inhibition of Chk1 can overcome the 4N arrest and generate cells with 2N DNA content. Our results suggest that this window of opportunity is most likely defined by p21 dependent APC activation. The point of no return may therefore be determined by the timing of substrate degradation by p21 dependent APC activation.

METHODS

Cell culture and synchronization

HCT116 (parental and isogenic $p21^{-/-}$) are diploid colorectal carcinoma cells and were kindly provided by Bert Vogelstein. In $p21^{-/-}$ HCT116 cells, p21 has been deleted by homologous recombination. HCT116 were grown in McCoy 5A medium with 10 % fetal calf serum[5]. Irradiation was delivered by a ^{137}Cs γ-irradiator at 2 Gray (Gy)/min. Where indicated, asynchronous cells were γ irradiated and 0.2 μg/ml nocodazole was added immediately to the culture. For synchronization with hydoxyurea (HU), cells were treated for 20 h with 2 mM HU. For synchronization by double thymidine block, cells were treated with 2 mM thymidine for 18 h, released for 6 h from the block, and then treated with thymidine for an additional 18 h. HU or double thymidine blocked cells were washed twice with PBS and drug-free medium, released for 2.5 h before γ irradiation (12 Gy) or adriamycin treatment (0.05 μg/ml).

Reagents

N-acetyl-leucyl-leucyl-norleucinal (LLnL), cycloheximide, caffeine, nocodazole, and roscovitine were dissolved in DMSO, HU and thymidine in McCoy 5A medium without serum, and adriamycin in water. All reagents were from Sigma. Cycloheximide was used at a concentration of 20 μg/ml to inhibit protein synthesis[43]. The IC_{50} of roscovitine for Cdk1-cyclin B and Cdk2-cyclin A is 0.45 μM and 0.7 μM, respectively, and 14-35 μM for Erk1/Erk2[31]. UO126 (Promega) was supplied in liquid form and frozen in aliquots. UCN-01 (7-hydroxystaurosporine) was provided by Robert J. Schultz (Division of Cancer Treatment

and Diagnosis, National Cancer Institute, USA). UCN-01 was dissolved in DMSO, aliquoted, and stored in 20°C.

Flow Cytometric Analysis

Cells detached by trypsinization were pooled with non-attached cells and stained with propidium iodide (PI) as a marker of DNA content. Where indicated, cells were analyzed by two-dimensional flow cytometry using MPM-2 as a mitotic marker and PI as described previously[44].

Cell extracts and antibodies

Cells were trypsinized, pooled with non-attached cells, washed with PBS and lysed with buffer containing 50 mM Tris, 0.5% NP-40, 150 mM NaCl, 50 mM sodium fluoride, 1 μm okadaic acid, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml each aprotinin and leupeptin. The addition of okadaic acid was found to be optional for extracts used for immunoblotting but was always included in extracts used for APC assays. After incubation for 30 min on ice, the extracts were centrifuged at 14,000 rpm in a microfuge for 20 min at 4°C and the supernatant collected. The following antibodies were used: Cyclin B1 (GNS1), Cdc20 (p55CDC, sc-1906), Vinculin (sc-5573), Chk1 (FL-476) and HRP-conjugated anti-goat IgG antibody (sc-2020) from Santa Cruz Biotechnology; Rb (554136) and p21 (556430) from Pharmingen; phospho-ERK1/2 (9101) from Cell Signaling; Mek2 (610235) and IAK (I71320) from Transduction laboratories. Polyclonal antibodies recognizing securin (J. A. Pintor-Toro) and Cdc27 (J-M Peters) were gifts. Other antibodies used were: phospho-histone H2AX (4411-PC, Trevigen), securin (Zymed), Plk1 (33-1700, Zymed), cyclin E (Clone 19A2, Oncogene), Cdh1 (MS-1116-PABX, Neomarker), actin (A2066, Sigma), HRP-conjugated anti-mouse (1050-05, Kirkegaard and Perry

Laboratories) and HRP-conjugated anti-rabbit (ALI3404, Biosource). Polyclonal Cdk2, Cdk1, cyclin A2 and Emi1 specific antibodies were as described[25,45]. Cyclin A2 was a gift from M. Ohtsubo and J.M. Roberts.

Kinase assays

Histone H1 kinase assays were performed using 100 μg cell extract as described previously[45].

For Plk1 kinase assays, 300 μg cell extract was incubated with 10 μl of packed protein A-agarose (Sigma) that had been pre-incubated with anti-Plk1 antibody. After 1 h at 4°C, the beads were washed 4 times with Plk1 IP buffer (50 mM Tris, 0.01% NP-40, 150 mM NaCl, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin and 5 μg/ml leupeptin) and once with Plk1 kinase buffer (10 mM Hepes pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA). The kinase assays were performed in 16 μl of kinase reaction mixture containing 166 μM ATP, 5 μCi [γ^{32}P]-ATP, 1 μg dephosphorylated casein (C8032, Sigma) and 10 μl packed protein A-agarose for 20 min at 37°C[46].

APC activity in cell extracts

APC^{Cdc20} or APC^{Cdh1} were prepared at indicated time points after irradiation by immunoprecipitation of cell extracts with anti-Cdc20 or Cdh1 antibody. Full-length human cyclin B cDNA and amino-terminal half of human Cdc20 cDNA encoding amino acids residues 1 to 250 were inserted into pET23d and expressed in the transcription-translation reticulocyte lysate system by Promega. Both recombinant proteins were purified by ProBondTM resin (Invitrogen). N-terminal half of Cdc20 and cyclin B were biotinylated with EZ-LinkTM Sulfo-NHS-LC-Biotin (Pierce). Active APC isolated with anti-Cdc20 or

anti-Cdh1 antibodies from extracts were incubated with biotinylated cyclin B and biotinylated Cdc20, respectively. The ubiquitination assay using biotinylated substrates were carried out for 60 min as described previously[29]. Briefly, the immunoprecipitates were incubated with 2 μg of biotinylated substrates in 30 μl of 5 mM Tris pH 7.5, 0.5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 2 mM creatinine phosphate, 1 μg/ml creatine phosphokinase, 0.2 μg/ml bovine ubiquitin, 40 μg/ml mouse recombinant E1 and 50 μg/ml human recombinant hE2-C. After the reaction, ubiquitinated substrates were re-purified using ProBond resin and were detected by immunoblotting with steptavidin-horseradish peroxidase.

BrdU staining

Cells grown on poly D-lysine coated coverslips were pulsed with 5 μM bromo-deoxyuridine (BrdU, Sigma) for 30 min prior to harvesting and processed for immunofluorescence as described previously[47].

RNA interference

siRNA duplexes were synthesized by Dharmacon (Lafayette, CO). Two Emi1 siRNAs duplexes AAA CUU GCU GCC AGU UCU UCA (Emi1 A) and AAG CAC UAG AGA CCA GUA GAC (Emi1 B) were synthesized[25]. Both Emi1 siRNA duplexes efficiently reduced the levels of Emi1. Four Rb siRNA duplexes AAA CAG AAG AAC CUG AUU UUA (Rb-1), AAG AUA CCA GAU CAU GUC AGA (Rb-2), AAG UUG AUA AUG CUA UGU CAA (Rb-3) and AAC CCA GCA GUU CGA UAU CUA (Rb-4) were synthesized. All four Rb siRNA duplexes efficiently reduced the levels of Rb. Chk1 siRNA duplex AAU CGU GAG CGU UUG AAC was used. Luciferase GL3 siRNA (CUU ACG CUG AGU ACU UCG A) was used as a negative control. RNA interference was performed as described by suppliers for 60 mm dishes using Oligofectamine (Invitrogen). Cells were transfected in

the absence of serum, and serum was added six hours later.

Quantitative real-time PCR.

mRNA expression levels were quantified by real-time quantitative PCR (ABI7900). 0.5 μg total RNA from each time point was reverse transcribed into cDNA using the Superscript II RNase H-Reverse Transcriptase Kit from Invitrogen. Validated Taqman primers (Applied Biosystems) for Emi1 were used for the PCR reactions and the quantitative measurements. The results were normalized to the observed value for glyceraldehyde-3-phosphate dehydrogenase.

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FIGURE LEGENDS

Figure 1. DNA damage induced activation of APC is p21 dependent.

(a) Degradation of APC substrates in γ irradiated $p21^{+/+}$ cells. $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells were γ irradiated (12 Gy) and treated immediately with nocodazole (0.2 μg/ml). The stability of APC substrates and the levels of other proteins after irradiation (IR) was examined by immunoblotting cell extracts with the indicated antibodies. Immunoblot with anti-actin antibody is shown as loading control.

(b) Irradiation induced activation of APC^{Cdc20} and APC^{Cdh1} in $p21^{+/+}$ cells but not in $p21^{-/-}$ cells. $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells were γ irradiated (12 Gy) and immediately treated with nocodazole (0.2 μg/ml). APC^{Cdc20} and APC^{Cdh1} were prepared at indicated time points after irradiation (IR). The activity of APC^{Cdc20} was determined using cyclin B as substrate (**upper panel**) while the activity of APC^{Cdh1} was determined using Cdc20 as substrate (**lower panel**) as described in Methods. Polyubiquitination of cyclin B (Ub-cyclin B) and Cdc20 (Ub-Cdc20) are indicative of APC^{Cdc20} and APC^{Cdh1} activity, respectively.

(c) Phosphorylation mediated electrophoretic mobility shift of Cdc27 occurs in both γ irradiated $p21^{+/+}$ and $p21^{-/-}$ cells. Cells were γ irradiated (12 Gy) and immediately treated with nocodazole (0.2 μg/ml). Cell extracts were analyzed by immunoblotting with polyclonal Cdc27 specific antibodies. The supershifted form of Cdc27 is indicated on the immunoblot.

(d) $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells were synchronized with HU, released for 2.5 h (0 h) and then γ irradiated (12 Gy). Cell extracts were examined for the stability of APC substrates (cyclin A2, cyclin B1, Cdc20 and securin) and the levels of other proteins at different times

after irradiation by immunoblotting with indicated antibodies. Mammalian somatic cells express only one cyclin A, cyclin A2, often referred to as cyclin A. Immunoblots with anti-actin and anti-vinculin antibodies are shown as loading controls.

(e) $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells were synchronized by double thymidine block, released for 2.5 h (0 h) and then γ irradiated (12 Gy). Cell extracts were examined for the stability of APC substrates and the levels of other proteins at different times after irradiation by immunoblotting with indicated antibodies.

(f) $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells were synchronized with HU, released for 2.5 h (0 h) and then treated with adriamycin (0.05 μg/ml). The stability of APC substrates (cyclin A2, cyclin B1, Cdc20 and securin) and the levels of other proteins in cell extracts prepared at different times after adriamycin (ADR) treatment are shown.

Figure 2. Inhibition of CDK activity activates APC in $p21^{-/-}$ cells after DNA damage.

(a) Suppression of Cdk1 and Cdk2 associated kinase activities after DNA damage is p21 dependent. $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). Cdk2 and Cdk1 associated kinase activities were measured in Cdk2 and Cdk1 immunoprecipitates (IP) of cell extracts prepared at different times after γ irradiation. Histone H-1 was used as a substrate.

(b) DNA damage induced association of Cdk2 and Cdk1 with p21. $p21^{+/+}$ HCT116 cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). Cdk1 and Cdk2 immunoprecipitates (IP) of cell extracts prepared at different times after irradiation were immunoblotted with p21 specific antibody.

(c) Roscovitine leads to the degradation of APC substrates in γ irradiated $p21^{-/-}$ cells. Cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). CDK activity was inhibited by 50 μM roscovitine (rosco) an inhibitor of Cdk1 and Cdk2 associated kinase activities. $p21^{-/-}$ cells were treated with roscovitine 10 h after γ irradiation. Cell extracts were examined for the degradation of APC substrates by immunoblotting with the indicated antibodies. Effect of roscovitine on Erk1/2 phosphorylation was tested using Erk1 and Erk2 phospho-specific antibodies (p-ERK) and is shown in the **lower panel**,

(d) APC substrate degradation in irradiated $p21^{-/-}$ cells is not due to inhibition of Erk1/Erk2. $p21^{-/-}$ cells synchronized and irradiated as in (**c**) were treated with 50 μM UO126 10 h after irradiation to prevent the activation of Erk1/Erk2. Cell extracts were examined for the degradation of APC substrates by immunoblotting with the indicated antibodies. Inhibition of Erk1/2 phosphorylation was confirmed using Erk1 and Erk2 phospho-specific antibodies (p-ERK).

(e) Roscovitine leads to the degradation of APC substrates in adriamycin treated $p21^{-/-}$ cells. $p21^{-/-}$ cells synchronized with HU were released for 2.5 h (0 h) and then treated with adriamycin (0.05 μg/ml). Roscovitine (50 μM final) was added 10 h after adriamycin treatment and cell extracts were examined for the degradation of APC substrates by immunoblotting with the indicated antibodies.

Figure 3. CDK inhibition prevents DNA synthesis and increase in ploidy of $p21^{-/-}$ cells after DNA damage.

(a) Cell cycle analysis of $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells after γ irradiation. Cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). Cells were stained with PI and MPM2 antibody at the indicated times and examined by flow cytometry. $p21^{+/+}$ cells arrest with 4N DNA content whereas $p21^{-/-}$ cells continue to cycle and generate cells with 8N DNA content. G1 DNA content is denoted as 2N.

(b) DNA synthesis in $p21^{+/+}$ and in $p21^{-/-}$ HCT116 cells after γ irradiation. DNA synthesis was measured by BrdU incorporation. Cells synchronized and γ irradiated as in (**a**) were pulsed with BrdU and stained with anti-BrdU antibody and PI as indicated in Methods. Data shown are mean ± SD of three different experiments.

(c) Percent of $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells with 8N DNA content at 48 h after γ irradiation. Five different experiments performed as in (**a**) are shown as mean ± SD.

(d) Roscovitine prevents the generation of 8N population of $p21^{-/-}$ cells after γ irradiation. Cells were synchronized with HU, released for 2.5 h (0 h) and then γ irradiated (12 Gy). Cells were treated with roscovitine (50 μM) 10 h after irradiation. Cells stained with PI at the indicated times were examined by flow cytometry. BrdU incorporation (DNA synthesis) was undetectable in irradiated $p21^{-/-}$ cells at 15, 24 and 48 h after treatment with roscovitine.

(**e**) Percent of $p21^{-/-}$ HCT116 cells with 8N DNA content after γ irradiation and roscovitine treatment. Data from 3 different experiments performed as in (**d**) are shown as mean ± SD.

Irradiated cells treated with roscovitine at 10 h after irradiation were examined at 48 h after irradiation.

(f) Roscovitine prevents the generation of 8N population of $p21^{-/-}$ cells after adriamycin treatment. $p21^{+/+}$ and $p21^{-/-}$ cells were synchronized with HU, released for 2.5 h (0 h) and then treated with adriamycin (0.05 μg/ml). Roscovitine (50 μM) was added to $p21^{-/-}$ cells 10 h after adriamycin treatment. Cells were stained with PI at the indicated times and examined by flow cytometry. Arrest of $p21^{+/+}$ cells with 4N DNA content is shown. BrdU incorporation was undetectable in adriamycin treated $p21^{-/-}$ cells at 15, 24 and 48 h after treatment with roscovitine.

Figure 4. Rb maintains APC activity in p21 dependent 4N arrest after irradiation.

(a) Down-regulation of Rb in $p21^{+/+}$ cells generates cells with 8N DNA content after DNA damage. $p21^{+/+}$ HCT116 cells were transfected with Rb siRNA (Rb-3) or control siRNA (Ctrl) 6 h prior to synchronization with HU. The cells were released from HU block for 2.5 h (0 h) and then γ irradiated (12 Gy). Cells were stained with PI at the indicated times and examined by flow cytometry.

(b) Down regulation of Rb leads to DNA synthesis in γ irradiated $p21^{+/+}$ cells. Percent BrdU incorporation from four different experiments performed as in (**a**) is shown as mean ± SD. Rb siRNA mediated decrease in Rb protein was verified in each experiment by immunoblotting.

(c) Percent $p21^{+/+}$ cells with 8N DNA content at 48 h after irradiation from six different experiments performed as in (**a**) are shown as mean ± SD.

(d) Down-regulation of Rb in $p21^{+/+}$ cells inactivates APC after irradiation. $p21^{+/+}$ HCT116 cells were transfected with Rb siRNA (Rb-3) or control siRNA (Ctrl) 6 h prior to synchronizing cells with HU. The cells were released from HU block for 2.5 h (0 h) and then γ irradiated (12 Gy). The stability of APC substrates and other proteins after irradiation were examined by immunoblotting cell extracts with the indicated antibodies.

Figure 5. p21 and Rb mediated down regulation of Emi1 protein and mRNA, respectively, activates APC after DNA damage.

(a) Decrease of Emi1 protein levels in $p21^{+/+}$ but not in $p21^{-/-}$ cells after DNA damage. $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy) (**upper panel**) or treated with adriamycin (0.05 μg/ml) (**lower panel**). Emi1 protein at different times after irradiation was examined by immunoblotting cell extracts.

(b) Emi1 degradation in $p21^{+/+}$ cells after DNA damage is proteasome dependent. $p21^{+/+}$ HCT116 cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated. Proteasome inhibitor LLnL (50 μM) was added at 9 h or at 18 h after irradiation and the cell extracts prepared 6 h later (15 h and 24 h after irradiation, respectively).

(**c**) The half-life of Emi1 protein is decreased after DNA damage. $p21^{+/+}$ cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated. Cycloheximide (CHX) was added at 0, 15 or 24 h after irradiation. Emi1 protein levels in cell extracts prepared at

different time after addition of CHX were analyzed by immunoblotting. Short and long exposures of the same immunoblot are shown.

(d) Failure to degrade Emi1 in $p21^{-/-}$ cells after γ irradiation is not due to the lack of Plk1 activity. $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). Plk1 associated kinase activity was measured in Plk1 immunoprecipitates (IP) prepared at different times after irradiation using de-phosphorylated α-casein as a substrate.

(e) Roscovitine leads to the reduction of Emi1 protein in γ irradiated $p21^{-/-}$ cells. $p21^{-/-}$ cells synchronized with HU were released for 2.5 h (0 h) and then irradiated (12 Gy). CDK activity was inhibited by treatment of cells with roscovitine (50 μM) 10 h after irradiation. Extracts were immunoblotted for Emi1 protein.

(f) The stability of Emi1 protein in asynchronous cells is regulated by CDK activity. Asynchronous $p21^{+/+}$ HCT116 cells were treated with roscovitine (50 μM). Cells extracts prepared at different times after the addition of roscovitine were immunoblotted for Emi1 protein.

(g) Reduction of mRNA levels of Emi1 in irradiated $p21^{+/+}$ HCT116 cells. $p21^{+/+}$ and $p21^{-/-}$ cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). At indicated times, mRNA was isolated and relative Emi1 mRNA levels were determined by quantitative real-time PCR. GAPDH was used as an internal control. Mean ± SD from four different experiments is shown. Emi1 transcript levels in $p21^{+/+}$ cells at 0 time were given an arbitrary value of 100%.

(h) Emi1 mRNA levels are dramatically reduced upon roscovitine treatment of irradiated $p21^{-/-}$ cells. $p21^{-/-}$ cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). The cells were treated with roscovitine (50 μM) 10 h after irradiation. Emi1 mRNA levels were determined by quantitative real-time PCR as in (**i**).

(i) Emi1 mRNA accumulates in irradiated Rb siRNA treated $p21^{+/+}$ cells. $p21^{+/+}$ HCT116 cells were transfected with Rb siRNA or control siRNA (Ctrl) 6 h prior to synchronizing cells with HU. The cells were released from HU block for 2.5 h (0 h) and then γ irradiated (12 Gy). Emi1 mRNA levels were determined by quantitative real time PCR as in (**h**). Mean ± SD from four different experiments is shown.

(**j**) Emi1 protein accumulates in irradiated Rb siRNA treated $p21^{+/+}$ cells. Rb and Emi1 protein levels in cell extracts from one of the four experiments in (**i**) are shown

(**k**) DNA damage induced generation of cells with 8N DNA content fails to occur in $p21^{-/-}$ cells with down regulated Emi1. $p21^{-/-}$ HCT116 cells were transfected with Emi1 siRNA (Emi1 B) or control siRNA (Ctrl) 6 h prior to synchronization with HU. The cells were released from HU for 2.5 h (0 h) and then γ irradiated (12 Gy). Flow cytometric analysis of cells transfected with Emi1 siRNA or control siRNA is shown. The percent 8N cells at 48 h is indicated.

(l) Down regulation of Emi1 prevents DNA synthesis in γ irradiated $p21^{-/-}$ cells. $p21^{-/-}$ HCT116 cells were transfected with the two different Emi1 siRNA (Emi1 A and B) or control siRNA (Ctrl) 6 h prior to synchronization with HU. The cells were released from HU

for 2.5 h (0 h) and then γ irradiated (12 Gy). Percent BrdU incorporation at different times after irradiation is shown.

(**m**) siRNA mediated down-regulation of Emi1 protein in $p21^{-/-}$ cells. Extracts prepared at zero time from cells in (**l**) were examined by immunoblotting.

Figure 6. DNA damage induced 4N arrest in $p21^{+/+}$ cells is dependent on ATR/ATM kinases.

(a) Treatment of γ irradiated $p21^{+/+}$ cells with caffeine results in the generation of cells with 2N DNA content. $p21^{+/+}$ HCT116 cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). Caffeine (5 mM) was added at 6 h or 15 h after irradiation. Cells were stained with PI at the indicated times and examined by flow cytometry.

(**b**) Treatment of γ irradiated $p21^{+/+}$ cells with UCN-01 results in the generation of cells with 2N DNA content. $p21^{+/+}$ HCT116 cells synchronized and irradiated as in (**a**) were treated with UCN-01 (100 nM) at 6 h after irradiation. Cells were stained with PI and examined by flow cytometry.

(**c**) $p21^{+/+}$ HCT116 cells synchronized and irradiated as in (**a**) were treated with UCN-01 (100 nM) at 15 h after irradiation, stained with PI and examined by flow cytometry.

(d) Irradiated $p21^{+/+}$ cells treated with caffeine or UCN-01 proceed through mitosis to generate cells with 2N DNA content. $p21^{+/+}$ HCT116 cells synchronized and irradiated as in (**a**) were treated with caffeine (5 mM) or UCN-01 (100 nM) at 6 h after irradiation. Cells were stained with PI and MPM2 and examined by flow cytometry.

(**e**) 2N cells generated by treatment of γ irradiated $p21^{+/+}$ cells with caffeine or UCN-01 do not cycle. $p21^{+/+}$ HCT116 cells synchronized and irradiated as in (**a**) were treated with caffeine (5 mM) or UCN-01 (100 nM) at 6 h after irradiation. Cells were stained with PI and examined by flow cytometry. See DNA profile in Supplementary Information, 1d for comparison.

(**f**) UCN-01 treatment at 24 h after γ irradiation does not lead to the generation of cells with 2N DNA content. $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). UCN-01 (100 nM) was added at 24 h after irradiation. Cells were stained with PI and examined by flow cytometry.

(**g**) Down regulation of Chk1 leads to the generation of cells with 2N DNA content after γ irradiation. $p21^{+/+}$ HCT116 cells were transfected with the Chk1 siRNA or control siRNA (Ctrl) at the time of release from HU. At 2.5 h (0 h) after release the cells were γ irradiated (12 Gy). Cells were stained with PI at different times after irradiation and examined by flow cytometry. Cell extracts were examined for Chk1 and cyclin B protein levels by immunoblotting.

(h) Treatment of γ irradiated $p21^{-/-}$ cells with caffeine does not prevent the generation of cells with 8N DNA content. $p21^{-/-}$ HCT116 cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). Caffeine (5 mM) was added at 6 h or 15 h after irradiation. Cells were stained with PI and examined by flow cytometry. The percent 8N cells at 48 h is indicated.

SUPPLEMENTARY INFORMATION

FIGURE LEGENDS

Figure S1.

(a) $p21^{+/+}$ HCT116 cells and $p21^{-/-}$ HCT116 cells were γ irradiated (12 Gy) and treated immediately with nocodazole (0.2 μg/ml). Cells were stained with PI examined by flow cytometry. Both $p21^{+/+}$ and $p21^{-/-}$ cells progress to 4N by 15 h after irradiation.

(b) Cell cycle analysis of $p21^{+/+}$ HCT116 cells synchronized with HU for 22 h and released into fresh medium. Cells were stained with PI and MPM2 at the indicated times and examined by flow cytometry.

(c) Cell cycle analysis of $p21^{+/+}$ HCT116 cells synchronized by double thymidine block and released into fresh medium. Cells were stained with PI and MPM2 at the indicated times and examined by flow cytometry.

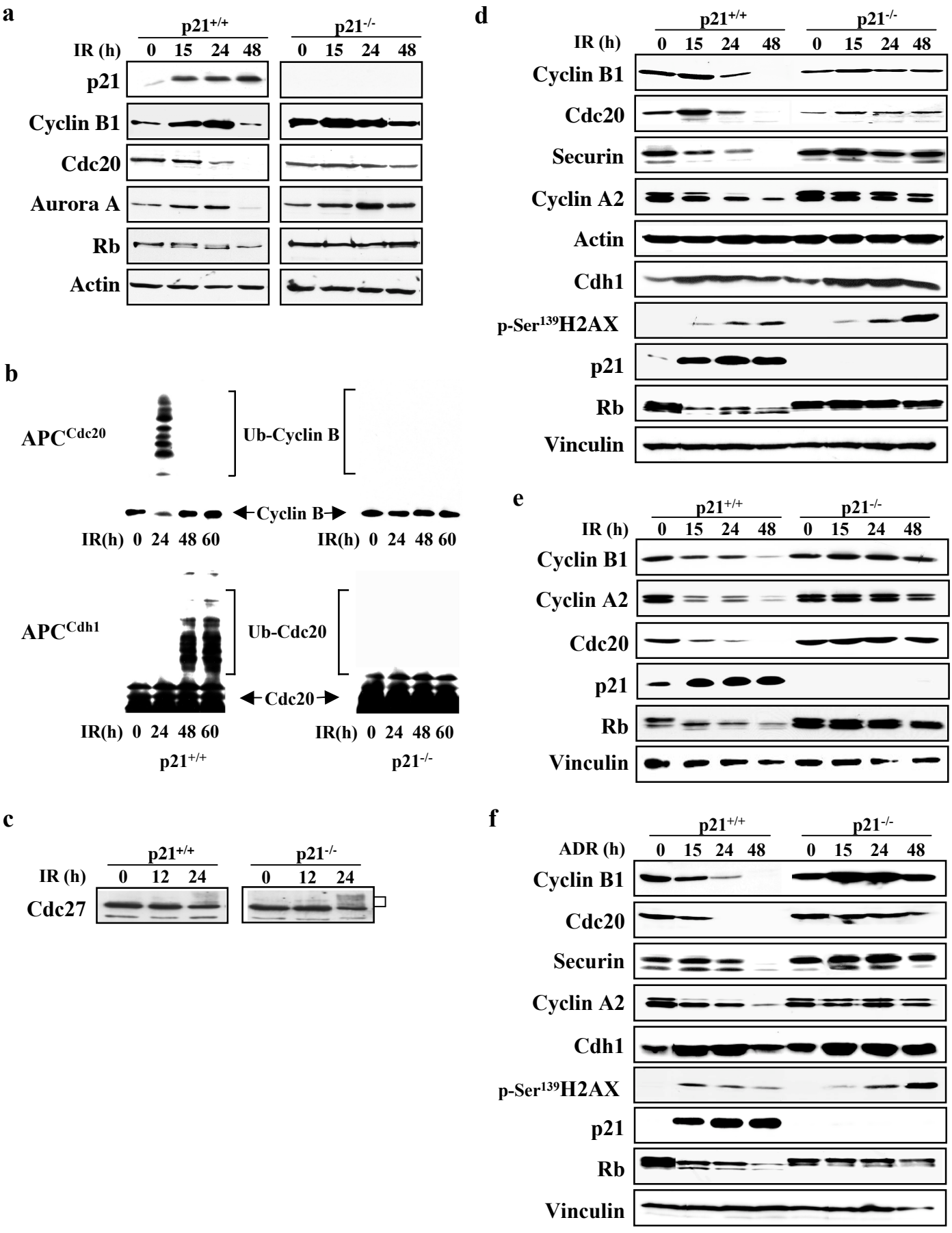
(**d**) $p21^{+/+}$ HCT116 cells released from HU block continue to cycle. $p21^{+/+}$ cells synchronized with HU for 22 h were released into fresh medium. Cells were stained with PI and MPM2 at the indicated times and examined by flow cytometry.

Figure S2. Emi1 protein levels remain unchanged in irradiated $p21^{-/-}$ cells treated with UO126. after DNA damage. Cell extracts from the experiment shown in **Figure 2d** were examined by immunoblotting with Emi1 specific antibodies. Briefly, $p21^{-/-}$ cells synchronized with HU were released for 2.5 h (0 h) and then γ irradiated (12 Gy). Cells were treated with 50 μM UO126 10 h after irradiation to prevent the activation of Erk1/Erk2.

Figure S3. $p21^{+/+}$ HCT116 cells synchronized by double thymidine block, released for 2.5 h (0 h) and then γ irradiated (12 Gy). Cells were treated with UCN-01 (100 nM) at 6 h after irradiation. Cells were stained with PI and examined by flow cytometry. Treatment of γ irradiated $p21^{+/+}$ cells with UCN-01 results in the generation of cells with 2N DNA content.

Figure S4.

Treatment of γ irradiated $p21^{+/+}$ cells with caffeine leads to degradation of APC substrates. Extracts prepared from the experiment shown in **Figure 6a** were examined for APC substrates (cyclins A2 and B1) and Emi1 by immunoblotting with specific antibodies.



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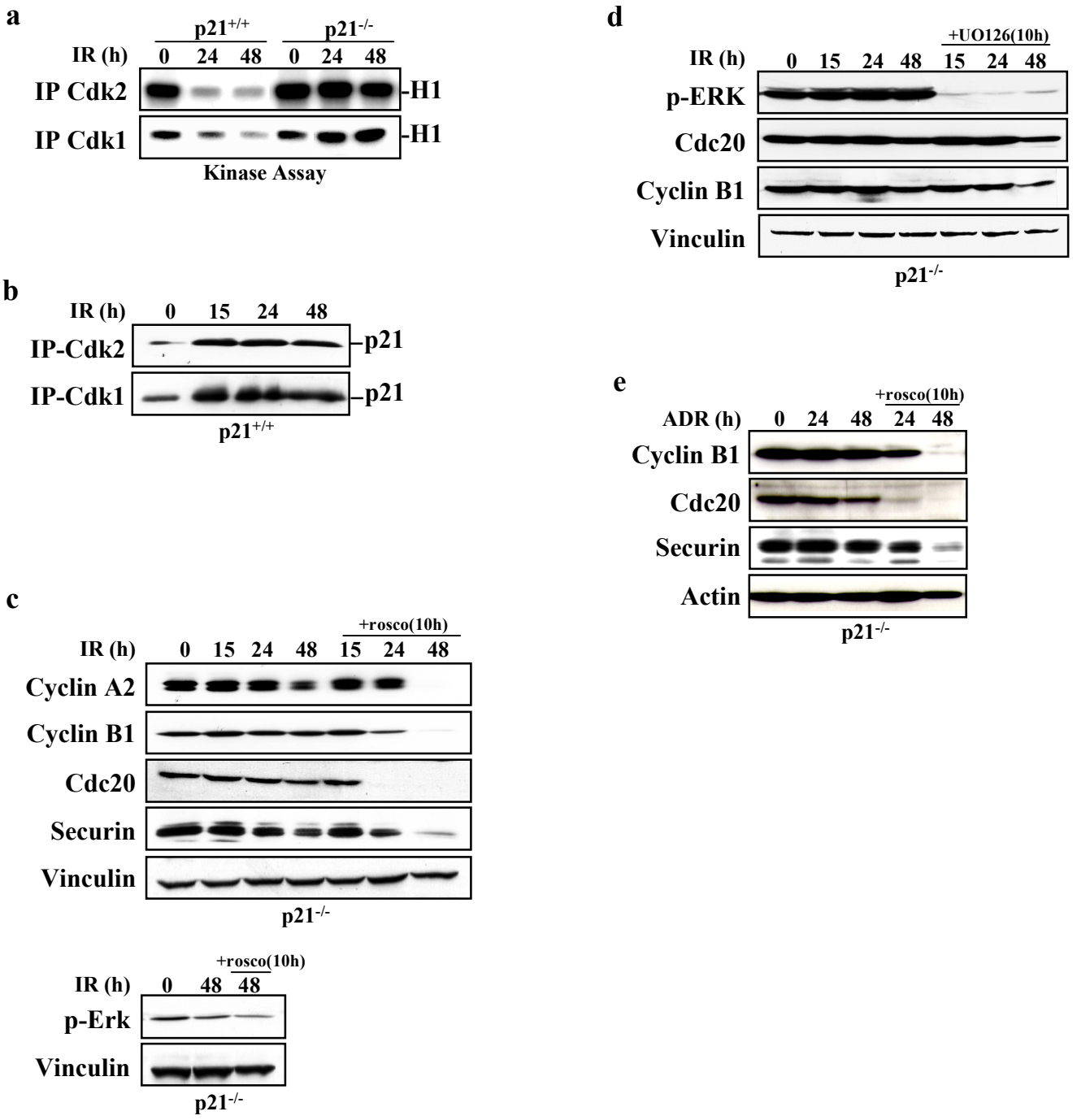

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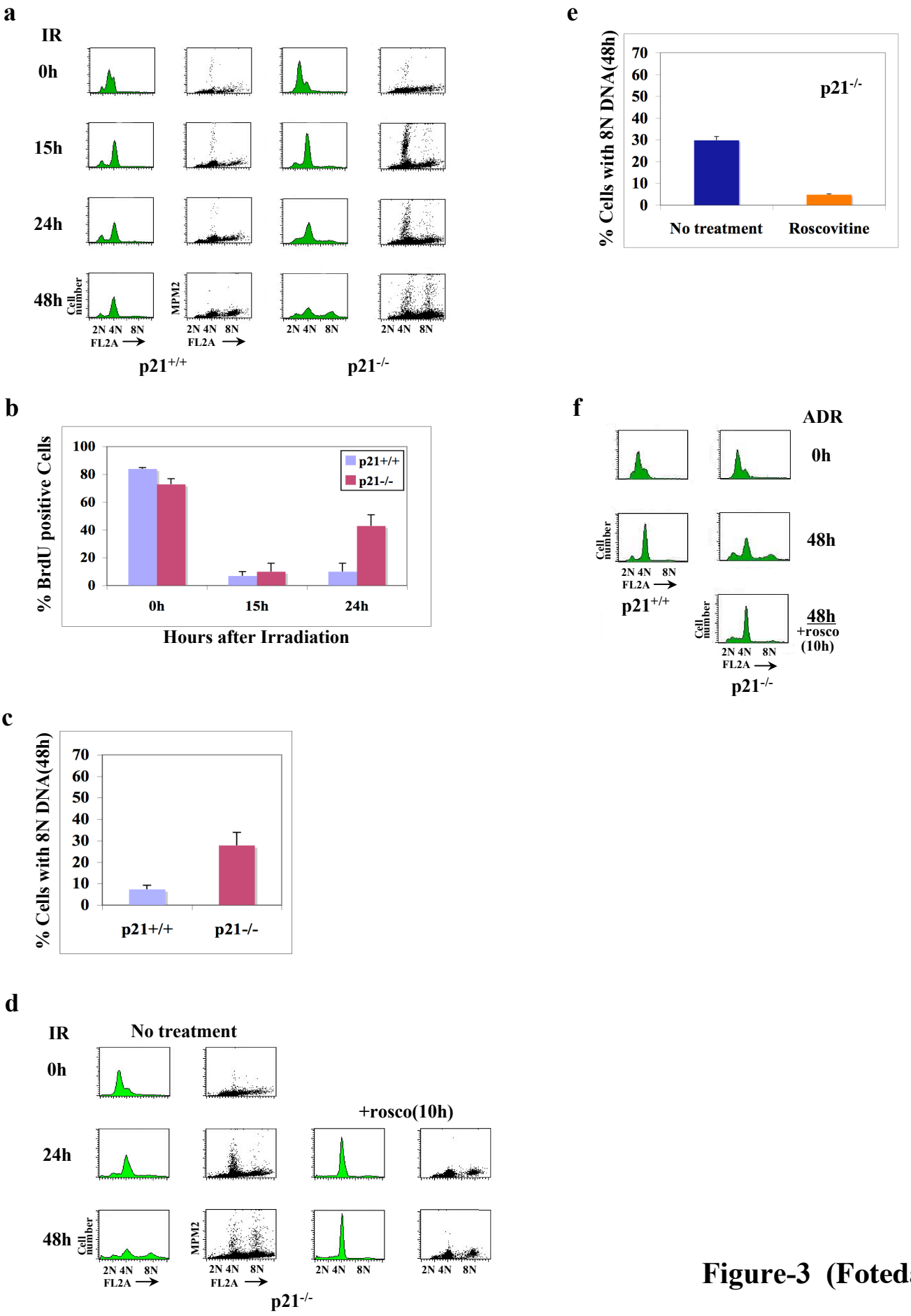

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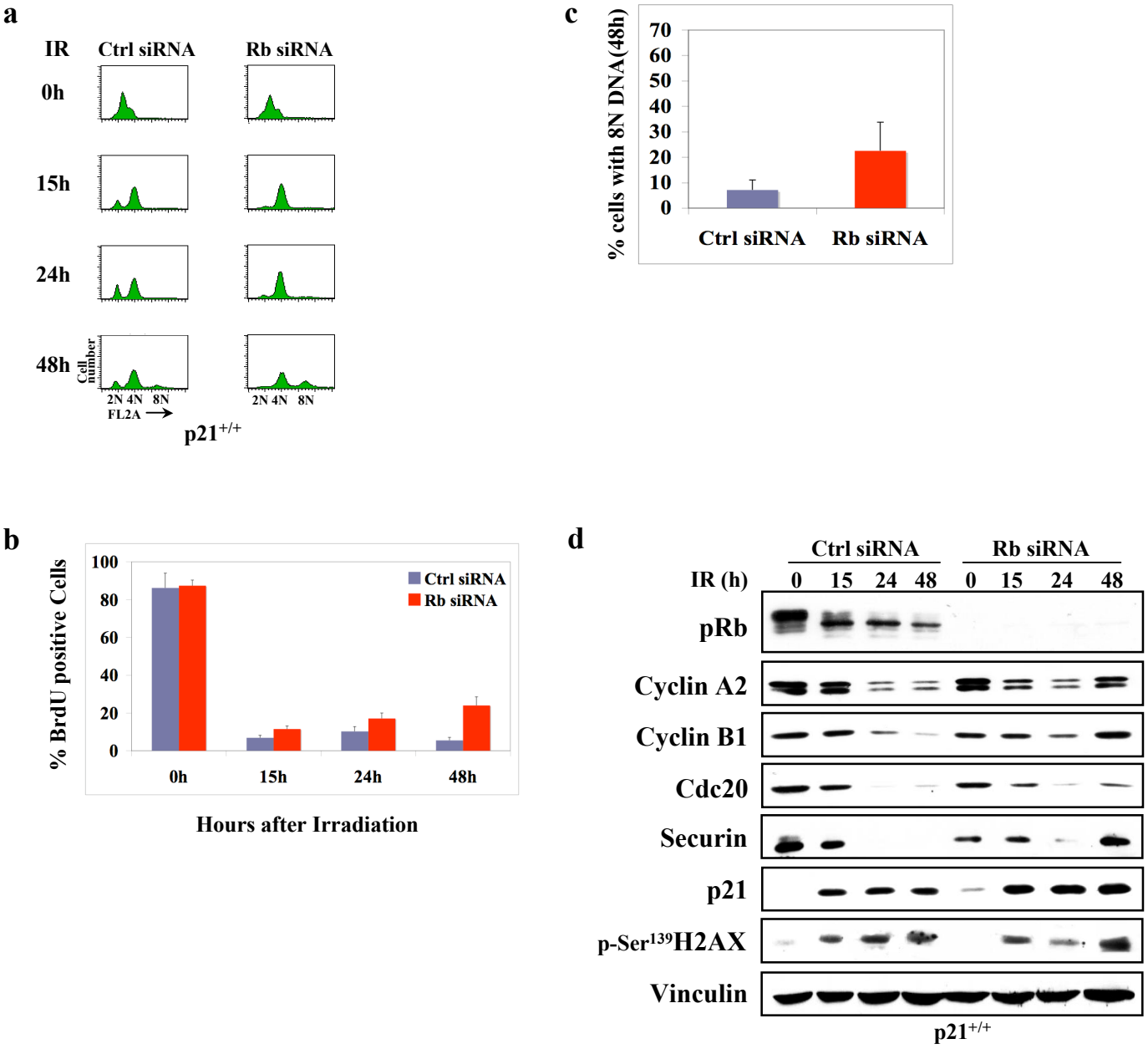

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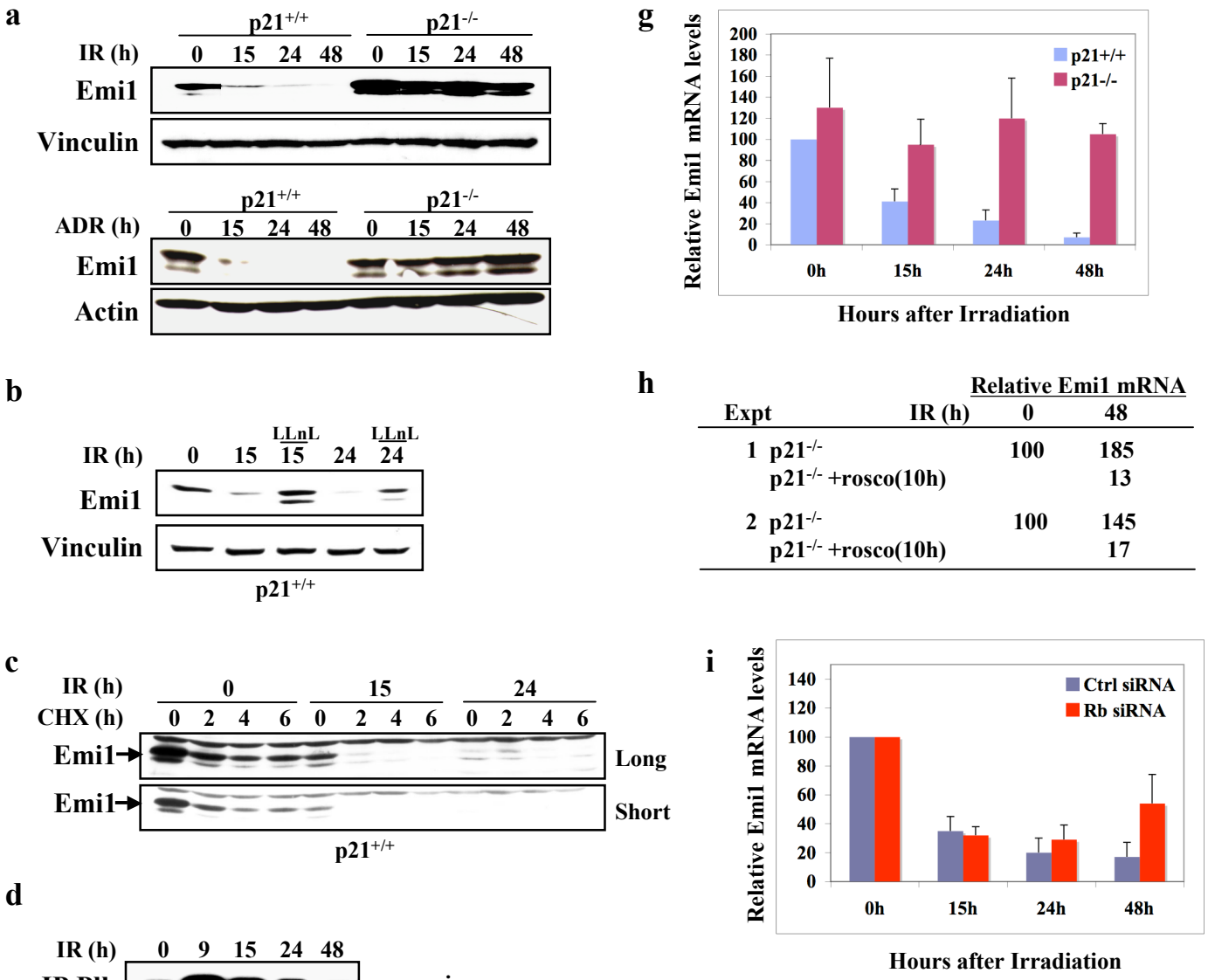
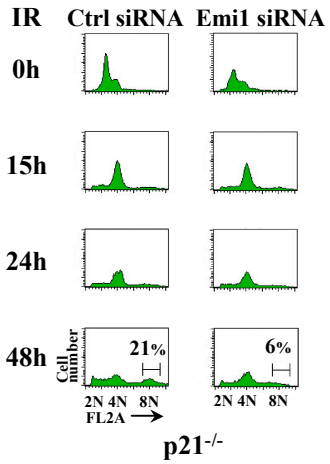


Figure-5 (Fotedar)

k



l

Expt	$p21^{-/-}$	IR (h)	%BrdU Positive cells 0	15	24
1	Control siRNA		60	12	30
	Emi1 siRNA A		64	8	19
	Emi1 siRNA B		72	9	11
2	Control siRNA		73		37
	Emi1 siRNA A		67		19
	Emi1 siRNA B		61		8

m

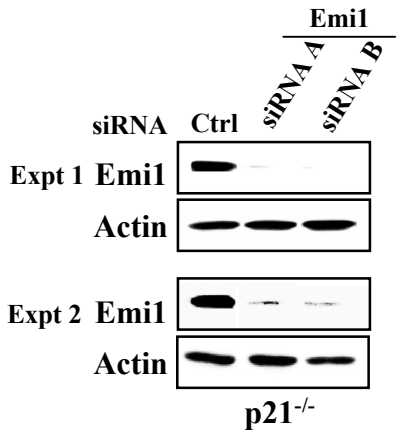


Figure-5 Continued (Fotedar)

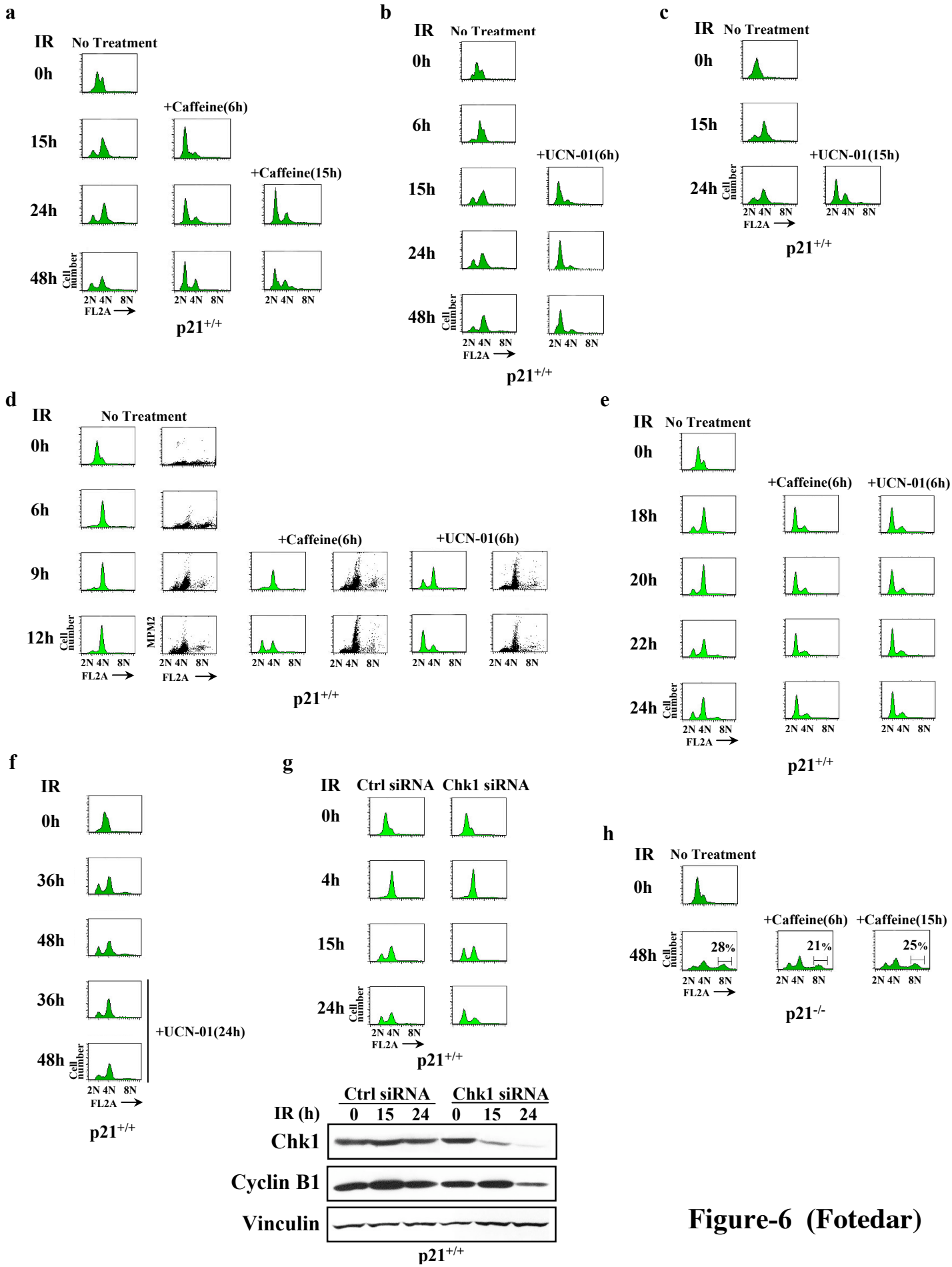
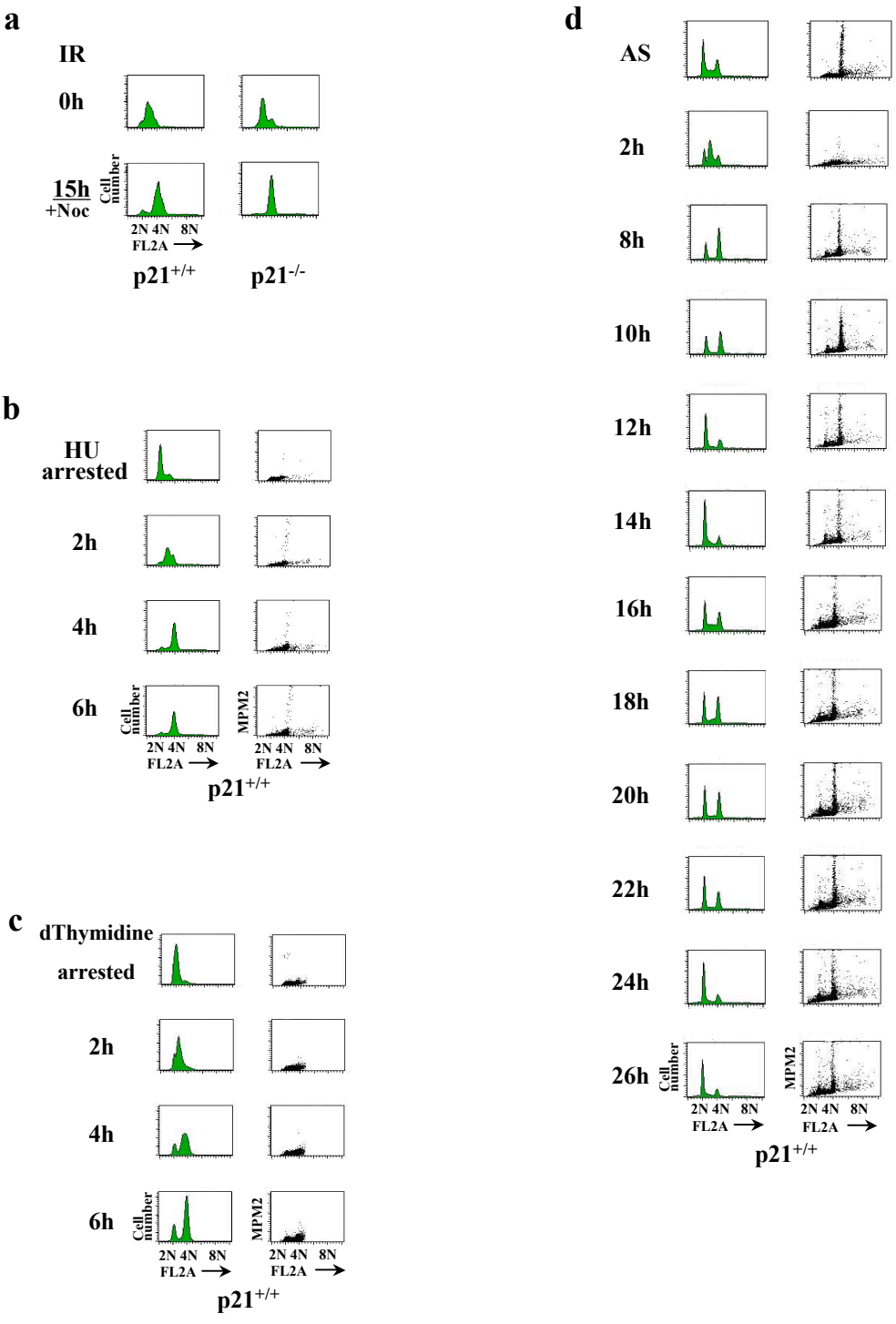
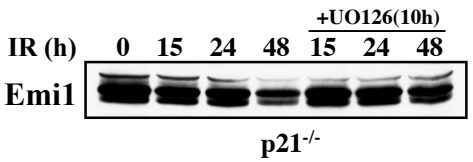


Figure-6 (Fotedar)

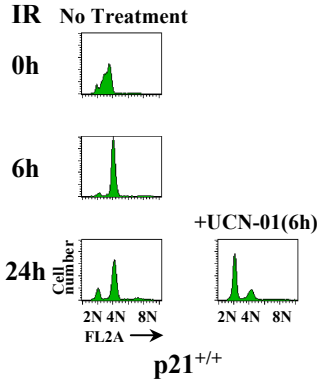
Supplementary Information, Figure 1.



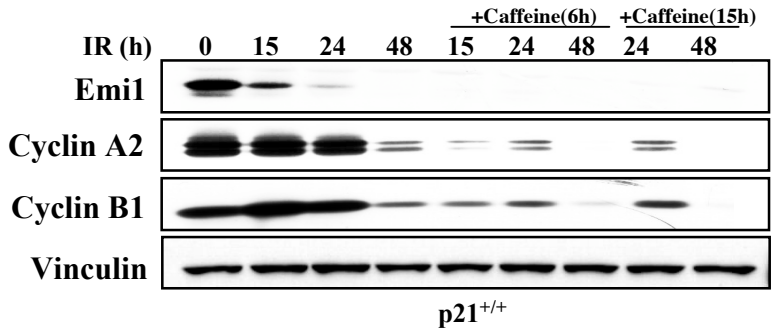
Supplementary Information, Figure 2.



Supplementary Information, Figure 3.



Supplementary Information, Figure 4.



DEUXIEME PARTIE : Résultats complémentaires

A. Supplementary figures

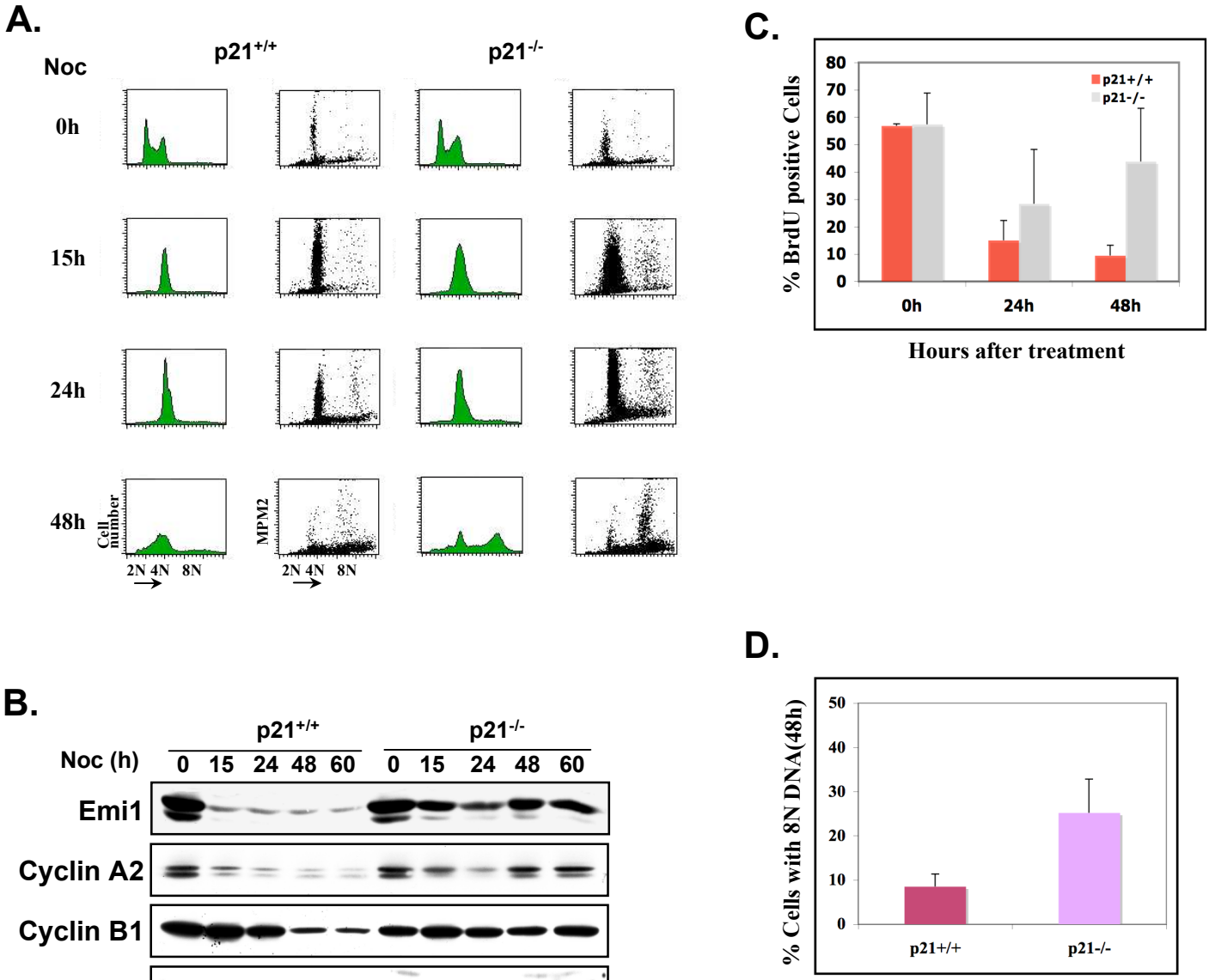
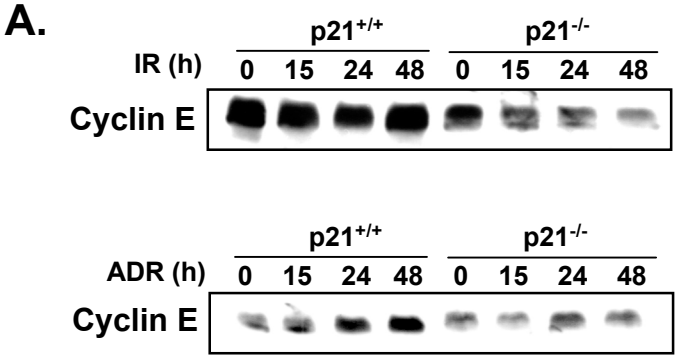

A.
Noc
p21+/+
p21-/-
0h
15h
24h
48h
Cell number
MPM2
2N 4N 8N
2N 4N 8N
B.
p21+/+
p21-/-
Noc (h)
0 15 24 48 60 0 15 24 48 60
Emi1
Cyclin A2
Cyclin B1
Cdc20
Securin
Rb
p21
Vinculin
C.
% BrdU positive Cells
80
70
60
50
40
30
20
10
0
p21+/+
p21-/-
0h
24h
48h
Hours after treatment
D.
% Cells with 8N DNA(48h)
50
40
30
20
10
0
p21+/+
p21-/-

Figure S1


A.
p21+/+
p21-/-
IR (h)
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Cyclin E
p21+/+
p21-/-
ADR (h)
0 15 24 48 0 15 24 48
Cyclin E

Figure S2

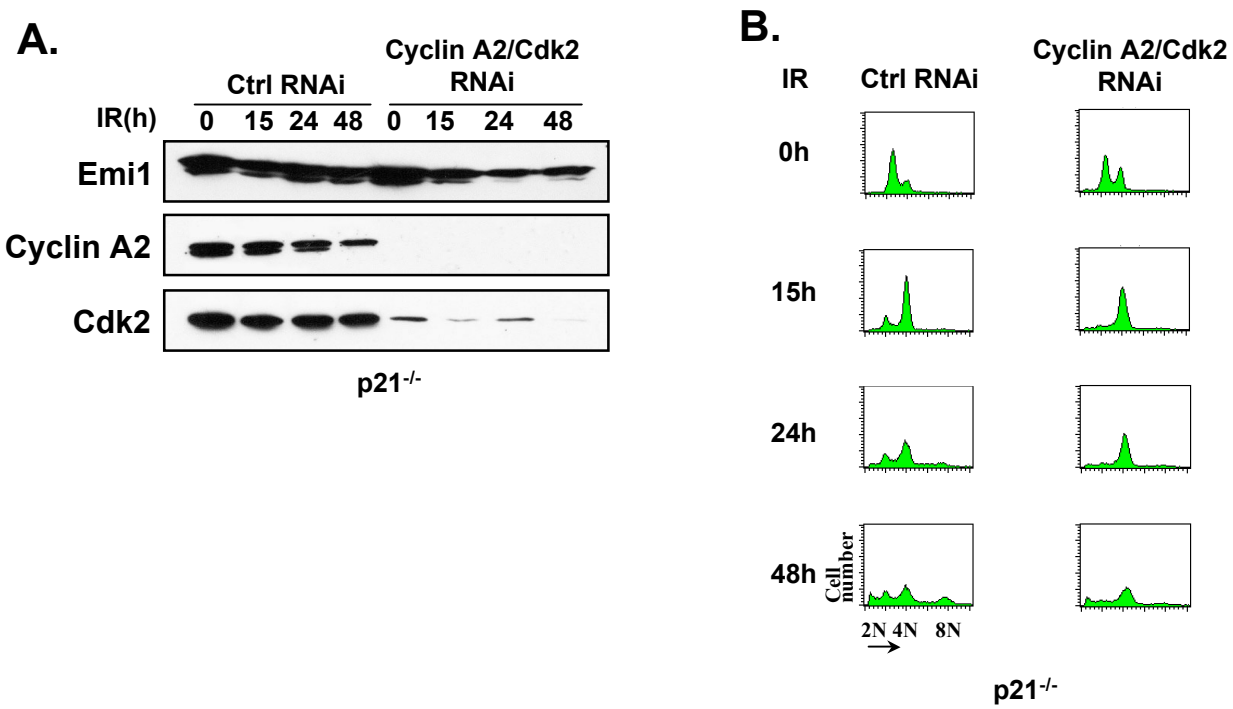

A.
Ctrl RNAi
Cyclin A2/Cdk2 RNAi
IR(h)
0 15 24 48 0 15 24 48
Emi1
Cyclin A2
Cdk2
p21-/-
B.
IR
Ctrl RNAi
Cyclin A2/Cdk2 RNAi
0h
15h
24h
48h
Cell number
2N 4N 8N
p21-/-

Figure S3

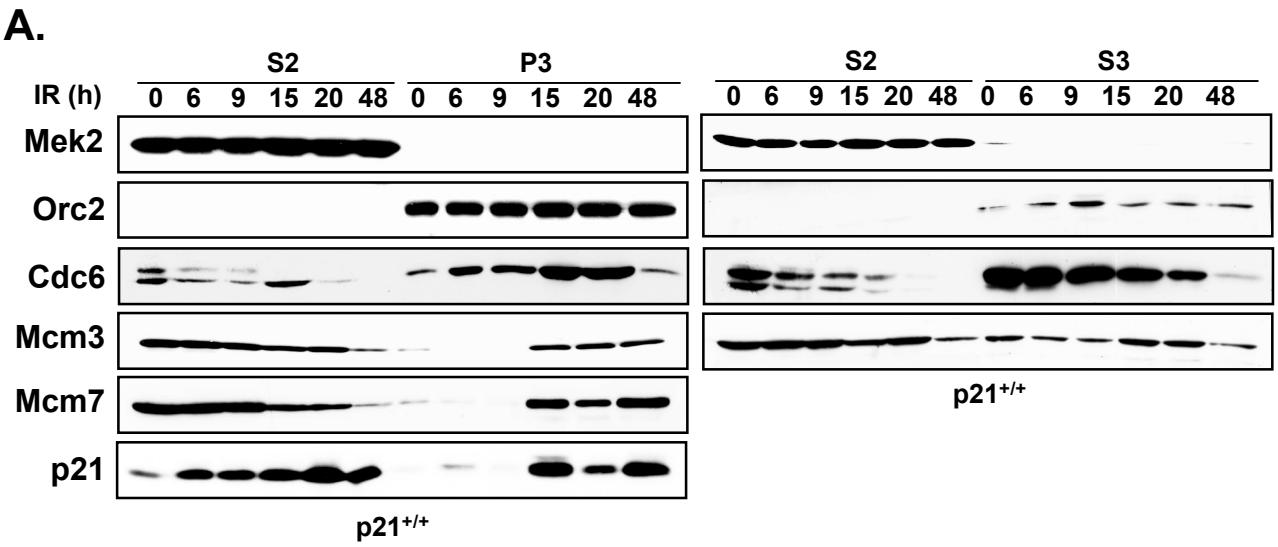
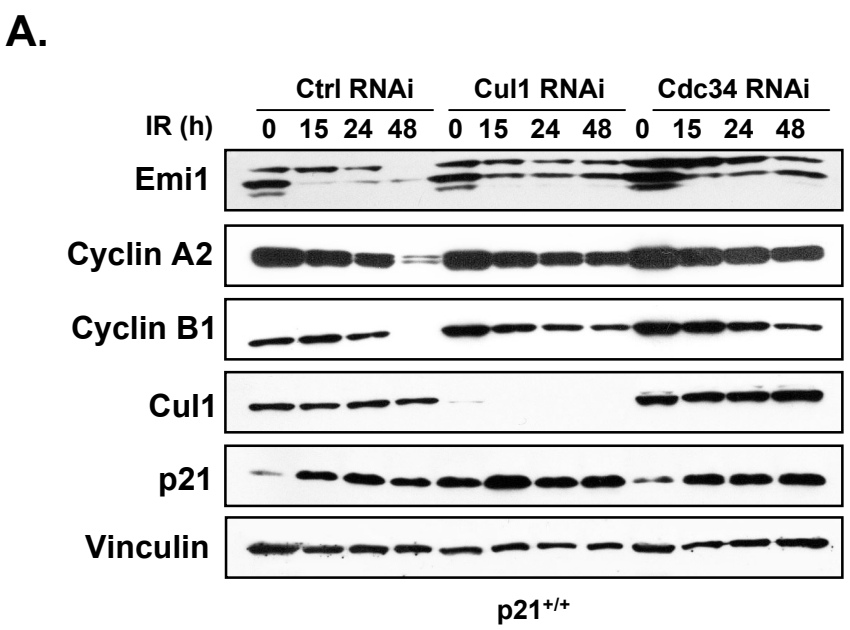

A.
S2
P3
S2
S3
IR (h)
0 6 9 15 20 48 0 6 9 15 20 48
0 6 9 15 20 48 0 6 9 15 20 48
Mek2
Orc2
Cdc6
Mcm3
Mcm7
p21
p21+/+
p21+/+

Figure S4


A.
Ctrl RNAi
Cul1 RNAi
Cdc34 RNAi
IR (h)
0 15 24 48 0 15 24 48 0 15 24 48
Emi1
Cyclin A2
Cyclin B1
Cul1
p21
Vinculin
p21+/+

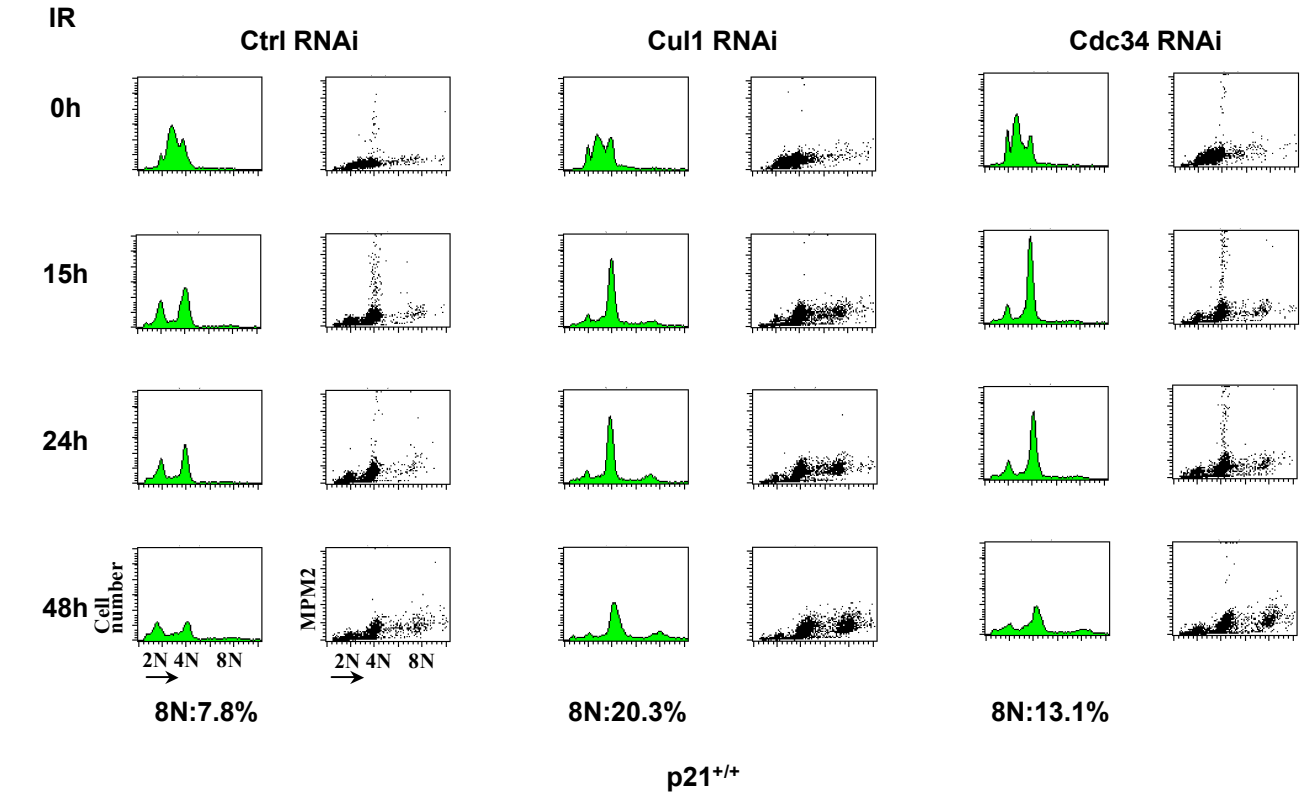

B.
IR
Ctrl RNAi
Cul1 RNAi
Cdc34 RNAi
0h
15h
24h
48h
Cell number
MPM2
2N 4N 8N
2N 4N 8N
8N:7.8%
8N:20.3%
8N:13.1%
p21+/+

Figure S5

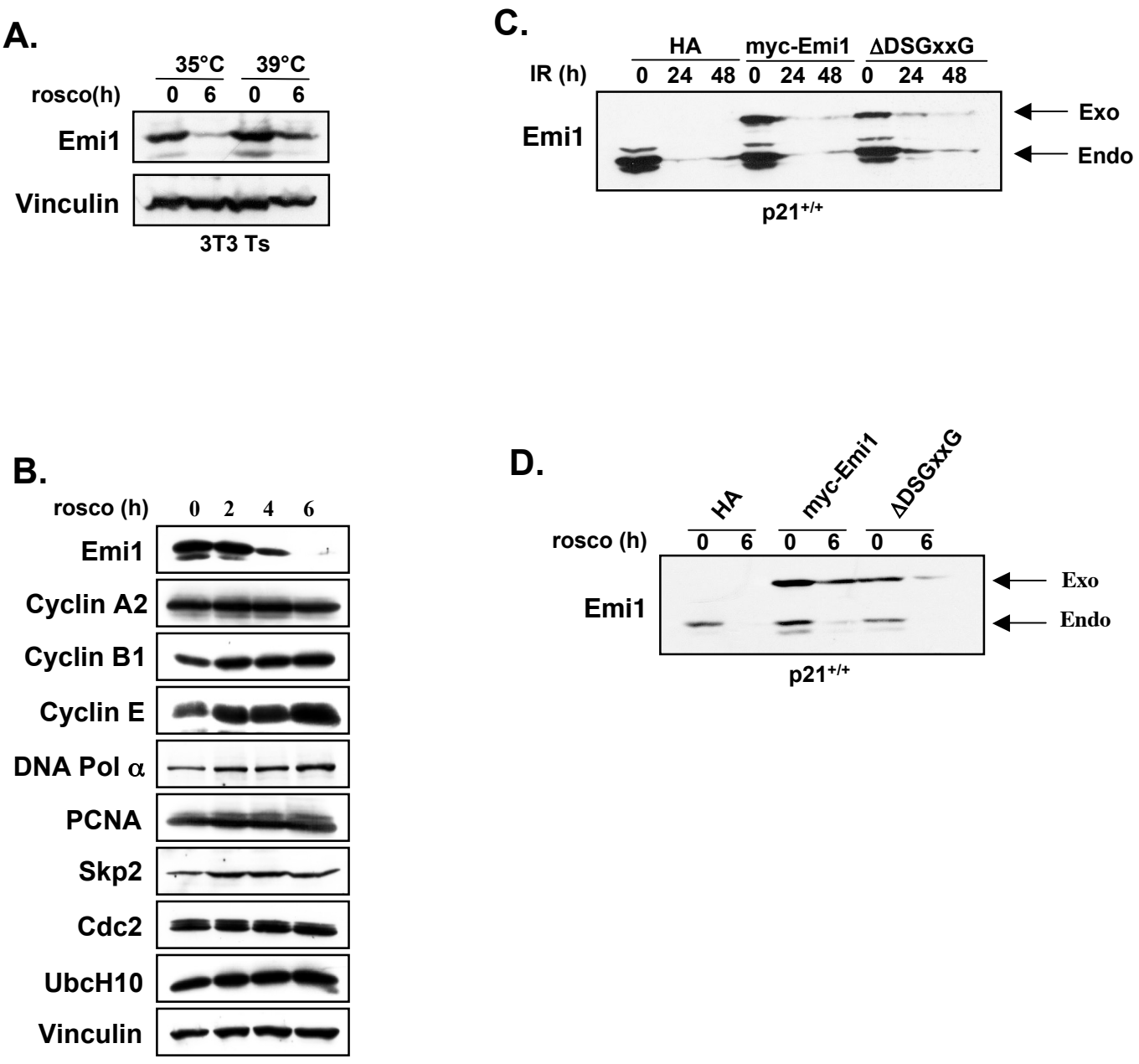

A.
35°C
39°C
rosco(h)
0 6 0 6
Emi1
Vinculin
3T3 Ts
C.
HA
myc-Emi1
ΔDSGxxG
IR (h)
0 24 48 0 24 48 0 24 48
Emi1
Exo
Endo
p21+/+
B.
rosco (h)
0 2 4 6
Emi1
Cyclin A2
Cyclin B1
Cyclin E
DNA Pol α
PCNA
Skp2
Cdc2
UbcH10
Vinculin
D.
HA
myc-Emi1
ΔDSGxxG
rosco (h)
0 6 0 6 0 6
Emi1
Exo
Endo
p21+/+

Figure S6

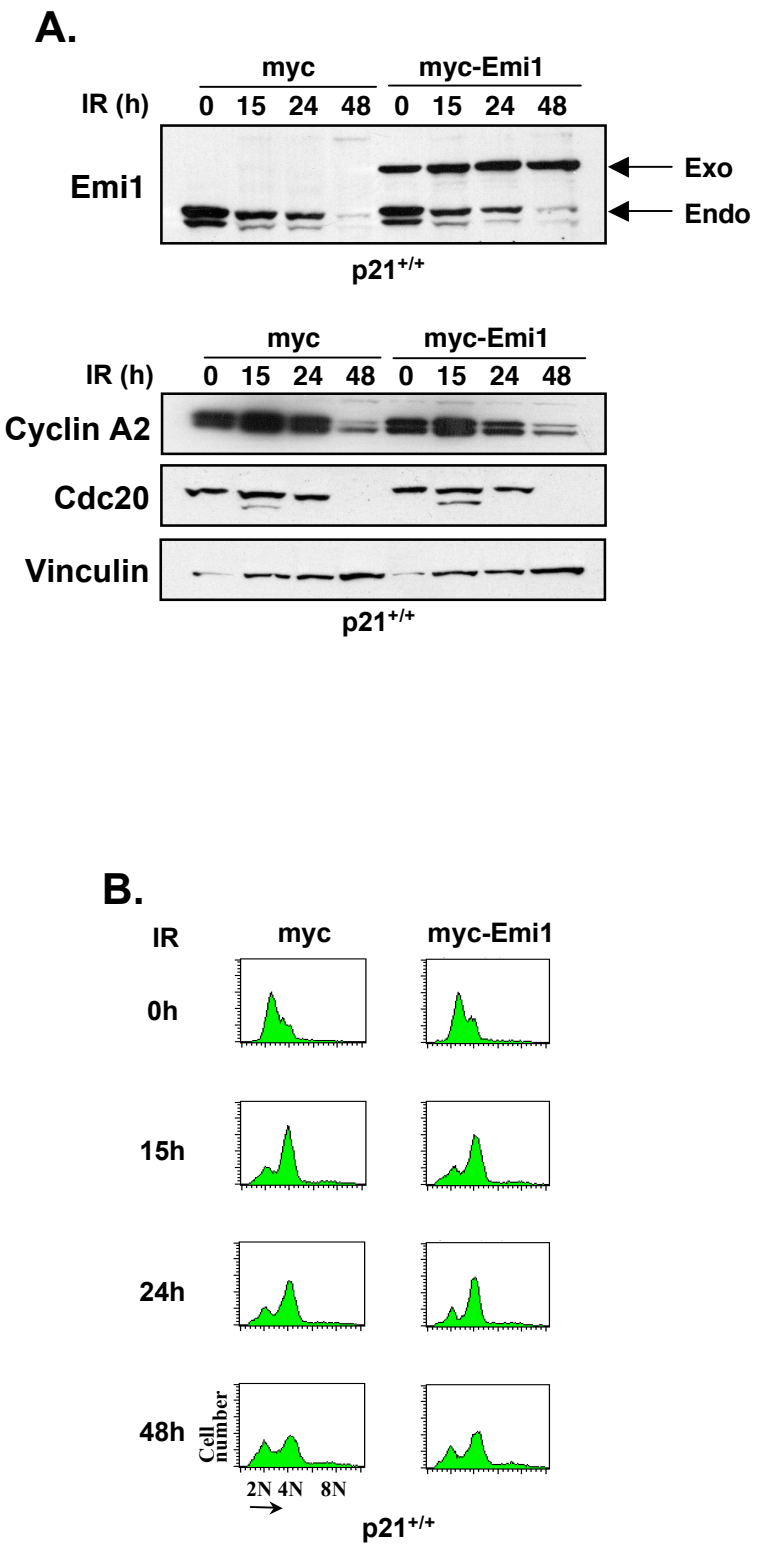

A.
myc
myc-Emi1
IR (h)
0 15 24 48 0 15 24 48
Emi1
Exo
Endo
p21+/+
myc
myc-Emi1
IR (h)
0 15 24 48 0 15 24 48
Cyclin A2
Cdc20
Vinculin
p21+/+
B.
IR
myc
myc-Emi1
0h
15h
24h
48h
Cell number
2N 4N 8N
p21+/+

Figure S7

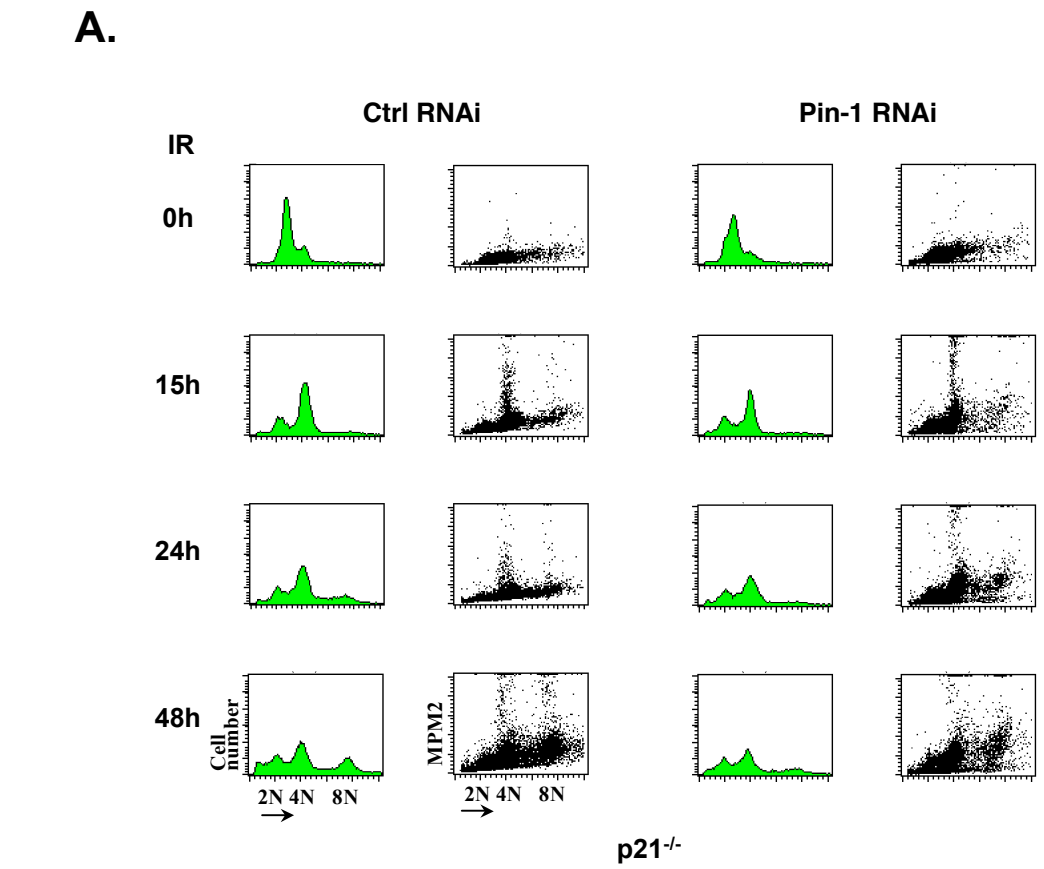

A.
Ctrl RNAi
Pin-1 RNAi
IR
0h
15h
24h
48h
Cell number
MPM2
2N 4N 8N
2N 4N 8N
p21-/-
B.
0h
48h
48h
Juglone, 5μM
48h
Juglone, 10μM
Cell number
MPM2
2N 4N 8N
2N 4N 8N
p21-/-

Figure S8

B. Supplementary figure legends

Figure S1. p21 prevents MTI-induced replication after failed mitosis through APC activation.
(A) HCT116 $p21^{+/+}$ and $p21^{-/-}$ cells were harvested and analyzed by flow-cytometry at the indicated times after treatment of nocodazole (0.2μg/ml). Cells were stained with propidium iodide for DNA content and MPM-2 antibody for mitosis indication. Asynchronous cells were at 0h. (B) Cell extracts from each time point were collected and the levels of indicated protein were analyzed by Western blotting. For loading control, vinculin was used. (C) DNA replication of cells treated with nocodazole was estimated using BrdU incorporation method and compared between HCT116 $p21^{+/+}$ and $p21^{-/-}$ cells. Cells were incubated with BrdU for 30 min and fixed with 2% paraformaldehyde. FITC-conjugated anti-BrdU antibody was used for staining. (D) The population of >4N DNA content was measured by flow-cytometric analysis and quantificated in the histograms.

Figure S2. Cyclin A / Cdk2 activity and mitotic delay.
(A) HCT116 $p21^{-/-}$ cells were transfected with 3 mixed RNAi oligos of Cyclin A (60nM each) and 2 RNAis of Cdk2 (40nM each) for 24h. Cells were blocked with HU for 20h, and then released for 2h. Cells were irradiated at 12Gy (0h), and total cell extracts were collected at indicated times and used for Western blotting. The levels of Emi1, Cyclin A2, and Cdk2 were analyzed. (B) Flow cytometry was used for cell cycle phase with propidium iodide and MPM-2 staining.

Figure S3. The levels of Cyclin E after DNA damage.
(A) HCT116 p21+/+ and p21-/- cells were blocked with HU for 20h. Cells were released for 2.5h and then irradiated at 12Gy (0h) or treated with adriamycin. Protein samples were collected at indicated times. The levels of Cyclin E were analyzed by western blotting.

Figure S4. The MCM helicase and Cdc6 loading onto chromatin after DNA damage.
(A) HCT116 $p21^{+/+}$ cells were blocked with HU for 20h, and then released for 2.5h and irradiated at 12Gy (0h). Protein samples were collected at different times by nuclear extract isolation method, and analyzed by Western blotting with antibodies of the indicated proteins. Mek2 and Orc2 were used for loading controls for S2 and P3, respectively.

Figure S5. Emi1 stabilization is Cul1-Cdc34 dependent after DNA damage.
(A) HCT116 $p21^{+/+}$ cells were transfected with Cul1 or Cdc34 RNAi oligos (180nM) for 24h, and then blocked with HU for 20h. Cells were released for 2h and irradiated at 12Gy (0h). The protein samples were collected at 0, 15, 24, and 48h. The levels of Emi1, Cyclin A2, Cyclin B1, Cul1, and p21 were analyzed by Western blotting. Vinculin was used for loading control. (B) DNA profile and MPM-2 staining were used for flow

cytometry analysis, and the progression to 8N DNA was monitored at 48h after DNA damage.

Figure S6. Emi1 degradation is dependent on Cdk activity and ubiquitin-activating enzyme (E1).
(A) Roscovitine, Cdk inhibitor, was added (50μM) to asynchronous HCT116 $p21^{+/+}$ cells (0h), and then protein samples were collected at 2, 4, and 6h after roscovitine treatment. The indicated antibodies were used for Western blotting to analyze the level of proteins. Vinculin was used for loading control. (B) Ubiquitin-activating enzyme (E1) mutant 3T3 temperature sensitive (*ts*) cells were incubated at 35°C. For the inactivation of E1 enzyme, cells were transferred and incubated at 39°C for 24h. Cells were treated with roscovitine (50μM) (0h), and then incubated for 6h. Protein samples were collected and analyzed by Western blotting with Emi1 antibody. Vinculin is shown as a loading control. (C) myc-tagged wild type Emi1 and DSGxxG dregon site mutated Emi1 plasmids were transfected (3μg mixed with 1μg of myc or HA empty plasmid, total 4μg of DNA) to obtain levels closer to endogenous Emi1 in HCT116 $p21^{+/+}$ cells. After 24h incubation, cells were blocked with HU for 20h. Cells were released for 2h and irradiated at 12Gy (0h). Protein extracts were collected at different times, and used for Western blotting to analyze the level of endogenous and exogenous Emi1. (D) Cells were transfected with same way as shown in (C). Roscovitine was added (50μM) in asynchronous HCT116 $p21^{+/+}$ cells (0h), and protein samples were collected 6h later. The turnover of exogenous and endogenous Emi1 was analyzed by Western blotting.

Figure S7. Continuous expression of Emi1 can not prevent the degradation of APC substrates after DNA damage.
(A) myc-tagged Emi1 plasmids were transfected (3μg mixed with 1μg of myc empty plasmid) to achieve a level closer to endogenous Emi1 in HCT116 $p21^{+/+}$. Cells were incubated for 24h, and then blocked with HU for 20h. After 2h release from HU, cells were irradiated at 12Gy (0h). At 15h, myc-tagged Emi1 plasmids were re-transfected for continuous expression. Total cell extracts were collected at different time point after DNA damage, and analyzed by Western blotting with Emi1, Cyclin A2, and Cdc20 antibodies. Vinculin was used for loading control. (B) FACS analysis was used for DNA profile with propidium iodide staining.

Figure S8. Pin-1 prevents Emi1 degradation for APC activation after DNA damage.
(A) HCT116 $p21^{-/-}$ cells were transfected with Pin-1 RNAi oligos for 6h, and then blocked with HU for 20h. Cells were irradiated at 12Gy after 2h release from HU (0h), and samples were collected at 15,24, and 48h after DNA damage. Cell ploidy was analyzed by flow cytometry with DNA profile and MPM-2 staining.

C. Supplementary Results

1. DNA replication after MTI-induced mitotic failure requires APC inactivation

In parallel with the response to DNA damage, we also investigated DNA replication after treatment with microtubule inhibitor (MTI), nocodazole. In the absence of p21, cells arrest in mitosis with 4N DNA contents after nocodazole treatment. However, long time exposure of MTI induces mitotic slippage and cells re-enter S phase after inappropriately exiting from mitosis (Figure S1A, S1C, S1D). In contrast, MTI-treated p21 wild type cells show increased level of p21 and persistent 4N DNA arrest and hypophosphorylation of Rb, which is thought to result from p21-associated CDK inhibition (Figure S1B).

MTI-induced endoreplication has been widely reported in p53, p21 or Rb-deficient cells (Cross et al., 1995; Di Leonardo et al., 1997; Khan et al., 1998; Stewart et al., 1999). The loss of p53 has been reported to promote chromosome instability and cause polyploidy (Shao et al., 2000; Vogelstein et al., 2000). Thus, it has been suggested that p53-dependent checkpoint pathway prevents rereplication through CDK inhibitor, p21.

We observed the differential regulation of Emi1 and degradation of APC substrates between wild type and p21-deficient cells (Figure S1B). In wild type cells, the level of Emi1 decreased in prophase and the APC appears to be activated through Emi1 degradation, which results in the degradation of APC substrates. In contrast, p21-deficient cell shows the re-accumulation of Emi1 and Cyclin A and this leads to APC inactivation and DNA replication after mitotic slippage. Taken together, these data suggest that DNA replication after mitotic failure may be due to APC^{Cdc20} activation through the inhibition of Emi1. Although we observe the degradation of Cyclin A in early mitosis (15h) in p21-deficient cells, APC largely remains inactivated at metaphase because of spindle checkpoint induced by MTIs as it is not sufficient to destroy other APC substrates, for example, securin, Cdc20 and Cyclin B (Figure S1B). While cells slip from mitosis, newly synthesized Emi1 and Cyclin A concomitant with Rb hypophosphorylation may inhibit the APC activity continuously in p21-deficient cell. In conclusion, our results suggest that p21 plays a pivotal role in APC activation through Emi1 and CDK inhibition in preventing MTI-induced DNA replication after mitotic failure.

2. Cyclin A-Cdk2 is required for DNA replication of p21-deficient cells after DNA damage

In mitosis, CDK inactivation allows dephosphorylation of CDK targets. This drives the events of late M phase and the low state of CDK activity prepares the cell for the next cell cycle with the assembly of pre-RC at replication origins (Diffley, 2004). APC activation is required for continuous destruction of cyclins to inactivate CDKs from mitosis to early G1. The inactivation of Cyclin A-CDK during mitotic exit is also mediated by CDK inhibitors, p21 and p27, or Rb family member, p107 (Chibazakura et al., 2004). Inactivation of Cyclin-CDK has been shown to be essential for the formation

of pre-RC complex. While low CDK activity promotes pre-RC assembly, CDK activitiy is required for the initiation of DNA replication in S phase. Either Cyclin A or Cyclin E activities can perform this function for replication of somatic cell. Cyclin E promotes pre-RC assembly. Cyclin A ends this assembly and initiates DNA replication (Coverley et al., 2002). In addition, the requirement of Cyclin E in DNA replication were shown in re-entering cell cycle from quiescence state (G0) and endoreplicative cells without intervening mitosis during embryogenesis (Geng et al., 2003; Mendez, 2003). Our results are in contrast with these studies as we show that although CDK activity remains high after DNA damage, DNA replication occurs after mitotic failure in p21-deficient cells.

It is still controversial whether Cyclin A-CDK activity is essential for replication after DNA damage. Cyclin A-Cdk2 has been described to promote replication by phosphorylating essential proteins. However, its function and target proteins for replicative initiation are mostly undiscovered in mammalian cells. Unlike in normal cell cycle, it is of interest that Cyclin A-CDK activity is required for prevention of rereplication in Emi1-depleted cells (Machida and Dutta, 2007). Moreover, Cyclin E-Cdk2 complex may have a role in rereplication in mice and in Emi1 depletion cells (Geng et al., 2003; Di Fiore and Pines, 2007). Our observations suggest that Cyclin A activity appears to be required for DNA replication after DNA damage. Previously, we observed that the level of Cyclin E (which is not an APC target) is downregulated in p21-deficient cells after DNA damage (Figure S2). In line with this, we propose that DNA damage-induced DNA replication in p21-deficient cells may require mostly Cyclin A activity but not Cyclin E activity.

We show that the inhibition of Cyclin A-Cdk2 by RNAi prevents DNA replication after DNA damage (Figure S3A and S3B). On the basis of these results, we suggest that Cyclin A-Cdk2 activity may be necessary for DNA replication after DNA damage. Previous studies have shown that the transient inhibition of Cyclin A induces mitotic entry delay and the overexpression of Cyclin A forces cells to enter mitosis earlier (Furuno et al., 1999; Mitra and Enders, 2004). We find that Cyclin A-Cdk2 RNAi treated cells enter mitosis and finally the premature inhibition of Cyclin A-Cdk2 prevents from entering next S phase after DNA damage.

3. Degradation of geminin is not essential for replication after mitotic failure

For the initiation of DNA replication, pre-RC complex is assembled with key regulators, including ORC, Cdc6, and Cdt1 onto the origin of DNA replication. Geminin is essential for controlling the origin licensing and is thought to play an important role in inhibiting Cdt1 activity. Cdt1 helps to load MCM complexes at replication origins (Wohlschlegel et al., 2000). Geminin has been also shown to stabilize Cdt1 in certain cell cycle context (Ballabeni et al., 2004). The degradation of geminin has been reported to occur at the end of mitosis and probably requires APC^{Cdh1} activity for its ubiquitination and degradation (Wohlschlegel et al., 2000; Tada et al., 2001). Recently, it has been demonstrated that geminin is required for preventing rereplication in human cells and in tumor cells (Zhu et al., 2004; Melixetian et al., 2004). Emi1-depleted cells undergo rereplication and show a reduction of geminin (Di Fiore and Pines, 2007). Interestingly, although Cdt1 levels are reduced in Emi1-depleted cells probably due to reduced geminin, rereplication occurs in

these cells. The Cdt1 is targeted for SCF^{Skp2} proteolysis by Cdk2 and Cdk4 during S phase (Liu et al., 2004; Nishitani et al., 2004) and high Cdk1 activity inhibits Cdt1 binding on the chromatin in murine cells (Sugimoto et al., 2004). We observed that the levels of geminin, Cdt1 and Cdc6 decrease in p21 wild type cells after DNA damage (data not shown). It has been shown that MCMs are loaded onto chromatin by Cdc6 and Cdt1 during mitosis. Consistent with these results, we find that MCM proteins and Cdc6 are loaded onto chromatin after DNA damage in p21 wild type cells yet replication does not occur (Figure S4).

It is possible that the origin firing does not occur due to the absence of Cyclin-CDK activity. In contrast to p21 wild type cells, we observe that these replicative factors remains unchanged in p21-deficient cells (data not shown). These results suggest that DNA replication after mitotic failure may occur in the presence of geminin. It is possible that geminin activity per se is downregulated in p21-deficient cells. For example, one report suggested that APC dependent ubiquitination of Xenopus geminin does not lead to proteolysis of geminin but inactivates it (Li and Blow, 2004). In this model CDK activity has a positive function in replication licensing process through the regulation of APC activity and the inactivation of geminin without its degradation. Non-proteolytic inactivation of geminin may allow Cdt1 stabilization. It therefore remains to be elucidated how DNA replication occurs in the presence of geminin and why high CDK activity does not downregulate Cdt1 in p21-deficient cells after DNA damage.

4. Emi turnover is dependent on Cul1, Cdc34 or Ubiquitin-activating enzyme (E1) after DNA damage

We have found that p21-dependent Emi1 turn over occurs by ubiquitination dependent pathway. We have tested whether Emi1 stability is Cul1 and Cdc34 dependent after DNA damage (Figure S5A). Cul1 is a core protein of SCF ubiquitin ligase (E3) complex. According to its structure, Cul1 is thought to contribute to catalysis of ubiquitination through the positioning of the substrate and the ubiquitin-conjugating enzyme (Zheng et al., 2002). Cdc34 is a homologue of E2 ubiquitin conjugating enzyme, which interacts with the F-box proteins (Winston et al., 1999). We observe that Emi1 is stabilized after DNA damage in p21 wild type cells transfected with Cul1 or Cdc34 RNAi. Cells undergo DNA damage-induced DNA replication (Figure S5B). These observations suggest that Cul1 and Cdc34 are partly implicated in Emi1 degradation after DNA damage. Moreover, we found that Emi1 is degraded after roscovitine treatment at the permissive temperature for E1 function (35°C) whereas Emi1 turnover dose not occur at non-permissive temperature for E1 function (39°C) in murine temperature sensitive E1 enzyme cell line (Figure S6A). We therefore conclude that Emi1 turnover is dependent on ubiquitination-proteasome pathway.

5. DNA damage-induced Emi1 turnover requires CDK inhibition but not Plk activity

It is suggested that Emi1 turnover is regulated by both mitotic CDKs and Polo-like protein kinase 1 (Plk1), which is activated in early mitosis by Cdk1 (Margottin-Goguet et al., 2003; Moshe et al., 2004). Plk1 kinase creates a phosphodegron, DSGxxG, which is recognized by the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase, and mediates the destruction of Emi1 in early mitosis (Hansen et al., 2004). To investigate the implication of Plk1 and β-TrCP in Emi1 turnover after DNA damage, we measured Cdk1 and Plk1 activity. Our results show that Plk1 activity remains high both in p21 wild type cells and in p21-deficient cells after DNA damage (Manuscript, Figure 5d). As we described before, Cdk1 activity is high in p21-deficient cells whereas it is low in p21 wild type cells (Manuscript, Figure 2a). In contrast with other studies, we conclude that high Cdk1 and Plk1 do not induce Emi1 degradation in p21-deficient cells. Furthermore, we observed that the downregulation of Plk1 by siRNA could not prevent the degradation of Emi1 after DNA damage in p21 wild type cells (data not shown). In addition, Emi1 is dramatically degraded after the treatment of CDK inhibitor, roscovitine, compared with other replicative proteins in asynchronous p21 wild type cells (Figure S6B). Proteasome inhibitor, LLnL, prevents its degradation, implying that CDK dependent degradation of Emi1 involves in ubiquitin-proteasome dependent proteolysis. However, the degradation of Emi1 after roscovitine treatment could not be prevented in Plk1 RNAi transfected cells (data not show). Thus, these results suggest that Emi1 turnover does not require Plk1 activity. These findings propose the intriguing possibility that multiple ubiquitination pathways are involved in Emi1 degradation. Our results bring up the question how CDK activity regulates the ubiquitin-dependent proteolysis of Emi1 after DNA damage.

To further examine whether Emi1 turnover after DNA damage is regulated by β-TrCP, we transfected wild type Emi1 or mutant Emi1 plasmids, in which the β-TrCP-recognized DSGxxG degron site is mutated. The proteins were expressed with closer to its endogenous level in p21 wild type cells. Interestingly, we observed that neither wild type Emi1 nor DSGxxG mutated Emi1 is stabilized after DNA damage in p21 wild type cells (Figure S6C). In parallel, we transfected these plasmids into asynchronous p21 wild type cells. CDK inhibition by roscovitine induces the degradation of exogenously expressed wild type Emi1 protein as well as the mutant Emi1 (Figure S6D).

6. DNA replication with mitotic failure requires not only Emi1-mediated APC inactivation but also continuous E2F transcriptional activity after DNA damage

The initiation of Cyclin A degradation requires the activation of APC^{Cdc20}, and it is mediated by Emi1 degradation in prophase. This model is supported by the evidence that non-degradable Emi1 prevents cell progression in prometaphase through the inactivation of APC (Reimann et al., 2001; Hsu et al., 2002). However, we could not observe any stabilization of Cyclin A upon Emi1 overexpression after DNA damage in p21 wild type cells (Figure S7A). Cells without stabilization of Cyclin A could not show DNA replication with mitotic failure after DNA damage although exogenous Emi1 was overexpressed (Figure S7B). Our data may correlate with a recent study that Emi1-mediated inactivation of APC is only needed in G2 (Di Fiore and Pines, 2007). This study suggested that Emi1-dependent APC inactivation does not affect the timing of Cyclin A degradation in prometaphase. In line with this, our results show that DNA

damage-induced DNA replication does not only occur through Emi1-dependent APC inactivation. It also requires Rb inactivation and E2F-mediated transcription.

7. Emi1 turnover after DNA damage appears to be involved in Pin1 activity

It is reported that Pin1 plays a role in the stabilization of Emi1 by preventing its degradation from $SCF^{\beta\text{-}TrCP}$ (Bernis et al., 2006). Pin1 is a peptidyl-prolyl cis/trans isomerase, which specifically binds to phosphorylated S/T-P dipeptides (Yaffe et al., 1997). It affects cell functions through isomerization of proteins by regulating enzymatic activity, protein stability or protein-protein interaction. CDK or MAPK phosphorylated target proteins, which are recognized by Pin1 isomerase. Pin1 associates with phosphorylated target protein and its binding may prevent the degradation of target protein.

In p21-deficient cells, DNA replication after mitotic failure requires Emi1 expression necessarily for APC inactivation. We have shown that CDK activities remain high after DNA damage in p21-deficient cells. In support of our postulation on the positive role of Emi1 for APC inactivation in G2 after DNA damage, we find that the inhibition of Pin1 by siRNA is capable of preventing replication after DNA damage in p21-deficient cells (Figure S8A). We also treated p21-deficient cells with Pin1 inhibitor, Juglone. Depending on concentration of juglone, generation of 8N is effectively prevented after DNA damage (Figure S8B).

D. Discussion

1. Regulation of ploidy through APC activity

The anaphase-promoting complex/cyclosome (APC) has a crucial role in the control of cell division during mitosis, and its activity is strictly regulated with the degradation of specific substrates in temporal and spacious control. This regulation of APC/C is achieved through participation of two activators, Cdc20 and Cdh1, and its inhibitor, Emi1.

a. p21-dependent Emi1 degradation mediates APC activation and regulates cell ploidy after DNA damage

Our study shows that p21 is implicated in the control of Emi1 turnover during cell cycle in response to DNA damage. We observed that DNA damage (γ-irradiation or adriamycin) in G2 leads to the activation of APC in p21 wild type cells. APC substrates are degraded following Emi1 destruction and cells arrest with 4N DNA content. In contrast, p21-deficient cells show stabilization of Emi1 and APC substrates and failure to activate APC after DNA damage. p21-deficient cells show failure of mitosis, delay in mitotic exit, and finally re-enter S phase to generate 8N. Moreover, in the absence of p21, the cell shows high CDK activities after DNA damage whereas it is low in p21 wild type cells. We postulate that the inhibition of CDK activity mediated by p21 is involved in Emi1 degradation after DNA damage. High CDK activity is required for DNA replication to keep the APC inactive (Amon et al., 1994; Zachariae et al., 1998; Jaspersen et al., 1999). To verify CDK activity involvement in Emi1 turnover, we treated p21-deficient cells with CDK inhibitor, roscovitine after DNA damage. We observe that CDK inhibition by roscovitine triggers Emi1 degradation and leads to the APC activation after DNA damage (Manuscript, Figure 2, 3 and 5). In addition, roscovitine treated p21-deficient cells were prevented from replicating their DNA after mitotic failure. Thus our results suggest that CDK activity is also indispensable for DNA replication after DNA damage.

Then, we investigated whether Emi1 stabilization is required for inactivation of APC and replication after mitotic failure in p21-deficient cells. The downregulation of Emi1 by RNAi induced the degradation of APC substrates after DNA damage (data not shown). This activation of APC by Emi1 depletion helps the cell to arrest with 4N DNA content and prevents from 8N generation (Manuscript, Figure 5k). We observe that Emi1-depleted cells release normally from HU block but do not enter mitosis after DNA damage. Subsequently, it is likely that the absence of Emi1 induces premature APC activation and destruction of its substrates, which causes cell cycle arrest. Together these results show that p21-dependent DNA damage-induced destruction of Emi1 is important to prevent the generation of polypoid cells. The continuous presence of Emi1 in irradiated p21-deficient cells compromises the APC activity through mitosis and G1. These results in the availability of APC substrastes needed to progress to mitosis and to replicate DNA

after mitotic failure in p21-deficient cells (Manuscript, Figure 3). The mechanism of Emi1 degradation by CDK inhibition remains to be further investigated.

b. Emi1 expression is regulated by Rb-E2F pathway after DNA damage

It has been proposed that the expression of Emi1 is transcriptionally controlled by E2F transcription factor (Hsu et al., 2002). Rb-E2F pathway also governs the expression of CDKs and other cyclins (DeGregori et al., 1995). As the cells progress from G1 to S phase, the phosphorylation of Rb by cyclin-CDKs has been reported to inactivate Rb (Hinds et al., 1992; Ewen et al., 1993; Kato et al., 1993). We observe that in p21 wild type cells, CDK inhibition by p21 triggers Emi1 degradation to initiate APC activation. Concomitant with p21 increase after DNA damage, Rb is dephosphorylated and downregulates Emi1 mRNA synthesis which results in maintenance of APC activity. Our results further show that the level of Emi1 mRNA is high in p21-deficient cells after DNA damage whereas it is low in p21 wild type cells (Manuscript, Figure 5g). The flow-cytometric analysis and Western blot results showed that the inhibition of Rb by RNAi in p21 wild type cells led to the progression to S phase following DNA damage. Emi1 appears to be partly stabilized after DNA damage in Rb-deficient cells (Manuscript, Figure 5j). The protein levels of Cyclin A decreased after DNA damage, consistant with E2F activation in Rb-deficient cells and lack of APC activity (Manuscript, Figure 4d). Furthermore, while Emi1 is degraded at early time, the timing of Emi1 accumulation in Rb-deficient cells co-relates with the entry of S phase in the presence of p21 after DNA damage (Manuscript, Figure 4). Hence we conclude that DNA replication after mitotic failure can be prevented through the inhibition of Rb-mediated Emi1 expression by p21. Functional inactivation of Rb has been shown to be involved in DNA damage-induced endoreplication in MEFs (Niculescu et al., 1998). Taking together these results, we conclude that Emi1 stabilization in Rb-deficient cells is required for inactivating APC to allow Cyclin A accumulation and replication of DNA after mitotic failure. However, it remains to be determined how Rb-deficient cells undergo DNA replication in the presence of p21, that is why CDK activity is not inhibited by p21 under these conditions. In conclusion, Rb may play a significant role in DNA replication by regulating Emi1 expression and maintaining of the activation of APC after DNA damage but not intiating APC activation (Figure 1).

2. Role of Chk1 in DNA damaged-induced mitotic arrest

DNA damage induces ATM-ATR signaling to activate DNA damage checkpoint through phosphorylation of Chk1 and Chk2 (Matsuoka et al., 1998; Liu et al., 2000). ATM/Chk2 lead to the activation of tumor suppressor protein, p53 through its stabilization (Canman et al., 1998; Banin et al., 1998). One study showed that Chk1 is involved in phosphorylation of p53 (Shieh et al, 2000). Previous studies have shown that Chk1 and not Chk2 plays a critical role in sustaining G2 DNA damage checkpoint (Zhao et al., 2002; Chen et al., 2003). Chk1 has been shown to affect Cdk1 inactivation indirectly through its negative regulation of Cdc25 and Plk1 (Tang et al., 2006). The

inhibition of Chk1 may help to bypass DNA damage pathway through inactivation of p53 and Cdk1 activation. Moreover, it has been described that p53 can repress transcriptionally Cyclin B and Cdk1 mRNA levels (Taylor et al., 1999; Innocente et al., 1999; Flatt et al., 2000). Thus, the stabilization of p53 by Chk1/Chk2 may play a role in mitotic entry delay. Our data suggest that DNA damage-induced 4N arrest can be overcome by ATM-ATR signaling inhibitor, caffeine, or Chk1/2 inhibitor, UCN-01. We also show that Chk1 appears to be principally implicated in this override (Manuscript, Figure 6). The inhibition of Chk1 by RNAi induces a rapid passage of p21 wild type cells through mitosis and exit of cells with 2N DNA content after DNA damage. UCN-01 treatment of p21 wild type cells also overcomes DNA damage response and the cells progress to G1 with 2N DNA (Manuscript, Figure 6 and p21 level not shown). The transient inhibition of Chk1 by RNAi does not show any significant change in p21 protein levels. These results imply that the inhibition of Chk1 may preferentially function to activate Cdk1 for mitotic exit and generation of cells with 2N DNA.

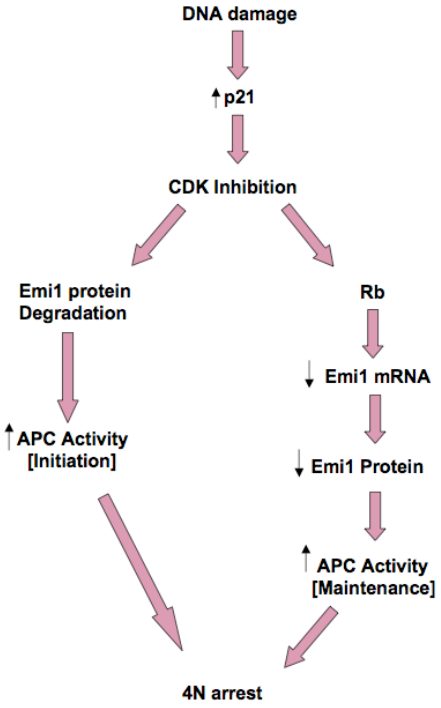


Figure 1. DNA damage response and APC activation

3. Conclusion

Recent studies have reported that Emi1 is involved in preventing endoreplication (Machida and Dutta, 2007; Di Fiore and Pines, 2007). These studies described the negative role of Emi1 in endoreplication. Whereas our data show that DNA replication after mitotic failure requires Emi1. Our results show that Emi1 stabilization can induce cyclin accumulation by inactivating APC and result in DNA replication after DNA damage. In parallel with the response to DNA damage, we also investigated DNA replication after treatment with microtubule inhibitor (MTI), nocodazole. MTI-induced replication after mitotic failure has been widely reported in p53, p21 or Rb-deficient cells (Cross et al., 1995; Di Leonardo et al., 1997; Khan et al., 1998; Stewart et al., 1999). We observed the differential regulation of Emi1 and degradation of APC substrates between wild type and p21-deficient cells. In wild type cells, the level of Emi1 is decreased in prophase and the APC appears to be activated through Emi1 degradation. APC substrates are destroyed and cells arrest in 4N DNA contents. In contrast, p21-deficient cell shows the re-accumulation of Emi1 and Cyclin A after mitotic slippage and this appears to be due to APC inactivation and leads to replication after mitotic failure.

We have shown that Rb, Cul1 or Cdc34 RNAi induces the stabilization of Emi1 after DNA damage in p21 wild type cells. These inhibitions allow accumulation of cyclins and generation of cell with 8N DNA content. However, it is of interest that DNA replication with low CDK activity after DNA damage occurs in the presence of p21. Because it is likely that CDK activity is essential for DNA replication after DNA damage, it remains to be determined how CDK acivity is increased in these RNAi experiments.

However, it is still unclear whether CDK activity is required for pre-RC formation after DNA damage. CDK activity keeps low when pre-RC is assembled on chromatin for replication licensing during mitosis and G1. Nevertheless, we observed that both Cdk1 and Cdk2 activities remain high after DNA damage and DNA replication occurs in p21-deficient cells. In our studies, DNA replication after DNA damage occurs presumably by Cyclin A activity and its associated CDKs. Nevertheless, it remains possible that DNA replication after mitotic failure under other conditions may require Cyclin E activity. Interestingly, we noticed that the level of Cyclin E was dramatically increased compared with Cyclin A in Rb-deficient cells. Our findings propose that Cyclin A-Cdk2 might be implicated in entering S phase after mitotic failure after DNA damage. Additionally, it is probably that the elevated level of Cyclin E provides an environment in which transcribed Emi1 is stabilized allowing Cyclin A to accumulate in Rb-deficient cells.
Together with these results, we postulate that the assembly of pre-RC after DNA damage may occur differently from normal mitotic process. The elucidation of its mechanism would be very important to understand the process of DNA replication in near future.

The initiation of Cyclin A degradation requires the activation of APC^{Cdc20}, and it is mediated by Emi1 degradation in prophase. This model is supported by the evidence that non-degradable Emi1 prevents cell progression in prometaphase through the inactivation of APC (Reimann et al., 2001; Hsu et al., 2002). However, we could not observe any stabilization of Cyclin A upon Emi1 overexpression after DNA damage in p21 wild type cell (Figure S7). Our data may correlate with a recent study that Emi1-mediated inactivation of APC is only needed in G2 (Di Fiore and Pines, 2007). This study suggested that Emi1-dependent APC inactivation does not affect the timing of Cyclin A

degradation in prometaphase. In line with this, our results show that DNA damage-induced DNA replication does not only occur through Emi1-dependent APC inactivation. It also requires Rb inactivation and E2F-mediated transcripton.

Lastly, we observed that the UCN-01 or inhibition of Chk1 abrogates mitotic arrest and generates cells with 2N DNA content after DNA damage in p21 wild type cells. It is interesting that the cells proceed to 2N despite DNA damage. It is probably that Chk1 is required for preventing mitotic failure after DNA damage. However, it remains to be determined how Chk1 is implicated in generation of 8N in p21-deficient cells after DNA damage.

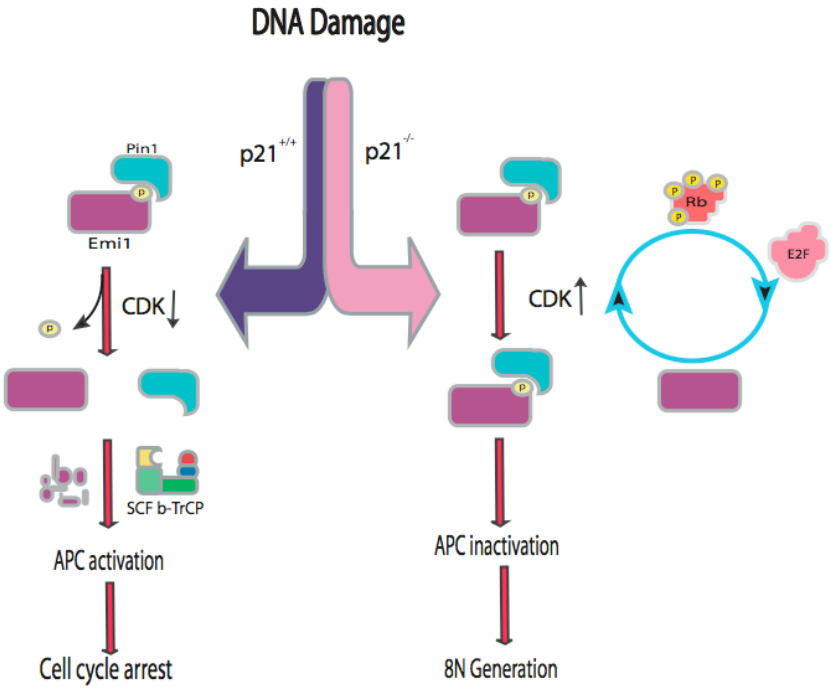


Figure 2. Possible mechanism of Emi1 degradation and APC activation after DNA damage.

In conclusion, our data support the model that p21 activity is required for DNA damage induced APC activation in G2. In p21-deficient cells, DNA replication after mitotic failure requires Emi1 expression necessarily for APC inactivation. In support of our postulation on the positive role of Emi1 for APC inactivation in G2 after DNA damage, we find that the inhibition of Evi5 or Pin1 is capable of preventing replication after DNA damage in p21-deficient cells (Figure S8) (data not shown for Evi5). However, the efficient inhibition of DNA replication occurs in Pin1 knockdown cells rather than in Evi5, implying that Pin1 during G2 is more effective in preventing the degradation of Emi1. Evi5 is thought to protect the centrosomal subpopulation of Emi1. In both HCT116

p21 wild type cells and p21-deficient cells, the protein levels of Pin1 were not changed after DNA damage (data not shown). In line with this, we postulate that Pin1 functions through the phosphorylation-dependent stabilization of its target protein Emi1 after DNA damage. As we have shown previously, CDK activities remain high after DNA damage in p21-deficient cells, but not in p21 wild type cells. It has been reported that Emi1 degradation is mediated by phosphorylation through mitotic CDKs and Plk (Margottin-Goguet et al., 2003, Hansen et al., 2004). Emi1 contains all five CDK phosohorylation sites and DSGxxxG domain for β-TrCP recognition. Pin1 binds to Emi1 and prevents the degradation through its isomerization on Ser10-Pro motif during G2 in *Xenopus* (Bernis et al., 2006). It is conceivable that Emi1 is kept in its phosphorylated form by high CDK activities after DNA damage and prevented from degradation through association with Pin1 (Figure 2). However, the identification of phosphorylation sites on Emi1 turnover still remains unclear and also the interaction of Pin1 with Emi1 after DNA damage needs to be further investigated in future studies.

The new finding of a role for p21 in Emi1 turnover provides new insight into the regulatory mechanisms responsible for CDK activity and APC activation in cell cycle. p21-dependent APC activation after DNA damage may be important in cell cycle arrest by preventing deregulation of uncoupled S/M process and chromosomal abnormalities.

MATERIEL & METHODES

Materiel & Methodes

A. Cell culture and protein extraction

HCT116 $p21^{+/+}$ and $p21^{-/-}$ cells were cultured at $10cm^2$ or $6cm^2$ dish in McCoy 5A midium with 10% FBS and incubated at 37°C. For HCT116 $p21^{-/-}$, cell was cultured in the presence of 0.4mg/ml Geneticin for cell selection and Geneticin was removed when cells were used for experiment.

Cells were collected by 1X EDTA-trypsinization and centrifuged at 1,000 rpm for 5 min. The pellet was washed once with PBS and re-collected by the centrifugation at 1,000 rpm for 5 min. The supernatant was removed and the cells were lysed with low salt concentrated lysis buffer (50mM Tris at pH8.0, 150mM NaCl, 0.5% NP-40, 5mM NaF, 1mM Na_3VO_4, 0.1mM PMSF, 50μg/ml of Leupeptin, 50μg/ml of Aprotinin. 1 μM Okadaic acid was added (optional) on the ice for 30 min. Cell debris were selected by high speed centrifugation at 13,000 rpm for 15 min and the soluble protein in the supernatant were collected.

B. Nuclear protein extraction & Chromatin isolation

Cells were trypsinized and collected by centrifugation at 1,000 rpm for 3 min. The pellet was washed with PBS, and centrifuged again at 1,000 rpm for 3 min. 150 μl of Buffer A (10mM HEPES pH7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.34M Sucrose, 10% Gylcerol, 1mM DTT, protease inhibitors (50mM NaF, 1mM Na_3VO_4, 1mM PMSF, 50μg/ml of Leupeptin, 100μg/ml of Aprotinin)) with triton-X (0.1%) was added and incubated on ice for 5 min. After centrifugation at 4,000-5,000 rpm for 5 min, the supernatant (S1) was centrifuged again at 20,000 rpm for 15 min in 4°C to preserve clear supernatant (S2). The pellet (P1) was resuspended for washing in 150 μl of Buffer A without triton-X, and collected by centrifugation at 4,500 rpm for 5 min in 4°C. Supernatant was removed and the pellet was incubated in 150 μl of Buffer B (3mM EDTA, 0.2mM EGTA, 1mM DTT, protease inhibitors) on ice for 30 min. After centrifugation at 4,500 rpm for 5 min in 4°C, the supernatant (S3) was preseved and the pellet (P2) was washed twice by 150 λ of Buffer B with centrfugation at 4,500 rpm for 5 min in 4°C. The supernatant was removed and the pellet was dissolved in laemmli buffer (50λ) and sonicated for 15 sec. After centrifugation at 14,000 rpm for 20 min in 4°C, the supernatant was collected (P3).

C. Antibodies

The following antibodies were used for immunoblotting : Rabbit anti-hEmi1 was a gift from Peter K. Jackson. Mouse monoclonal anti-hEmi1, securin, Plk1 were from Zymed. Monoclonal mouse anti-cyclin B1(GNS1), rabbit anti-Vinculin (H-300), rabbit anti-Actin, goat anti-Cdc20 (p55CDC), mouse anti-Chk1 (FL-476) were purchased from Santa Cruz Biotechnology. Monoclonal mouse anti-p21 and mouse anti-phospho-Rb were from Pharmingen. Rabbit anti-Phospho-cdc2 (Thr161), rabbit anti-phospho-Cdk2 (Thr160), and phosoho-ERK1/2 were from Cell Signaling technology. Mek2 and IAK were from Transduction laboratories. Anti-Cyclin A2, anti-Cdc2, anti-Cdk2, and anti-Cdk4 were raised from rabbit. Polyclonal anti-rabbit securin (J. A. Pintor-Toro) and Cdc27 (J-M Peters) were gifts. Cyclin A2 was a gift from M. Ohtsubo and J. M. Roberts. HRP-conjugated secondary antibodies were purchased from Kirkegaard and Perry laboratories / Southernbiotech (goat anti-mouse) and Biosource (goat anti-rabbit). FITC-conjugated secondary antibody was from Uptima (donkey anti-mouse). Other antibodies were: mouse anti-Cdh1 Ab-1 (MS-1116-PABX, Neomarkers), rabbit anti-phospho-histone H2AX (Trevigen), mouse anti-cyclin E (Clone19A2, Oncogene), monoclonal mouse anti-phospho-Ser/Thr-pro, MPM2 (Upstate).

D. Western blotting

The proteins were separated on polyacrylamide gel (SDS-PAGE) and transferred onto nitrocellulose membranes by semi-dried electrophoretic method. The membrane was blocked with 4% milk in TBS-T (10mM Tris pH7.5, 150mM NaCl, 0.1% Tween-20) for 1h at room temperature and incubated overnight at 4°C with primary antibody diluted in 0-4% milk. The membrane was washed 3 times with TBS-T for 10 min each and incubated for 2hr with HRP (horseradish peroxidase)-conjugated secondary antibody diluted at 1 :2000~1 :5000 in 4% milk. After 3 time washes with PBS for 30 min, the membrane was developed with ECL (Electrochemiluminescence) solution (SuperSignal West Pico Luminol, Pierce) as instructed by the supplier and the film (Hyperfilm, Amersham) was developped in the dark room.

E. Drugs used for Cell Cycle Analysis

1. Nocodazole

Nocodazole is a drug of benzimidazole carbamate family which integrates with β-tubulin subunit and inhibits the polymerization of microtubules. In weak concentration (50nM), nocodazole acts essentially on microtubule dynamic by inhibiting its elongation without global modification of microtubule mass (Jordan et al., 1992). In the range of 0.5 - 1μM concentration, the microtubules are massively depolymerized.
Nocodazole is used for the mitotic cell synchronization. As a result, it arrests the cells in metaphase by a spindle checkpoint.

2. Hydroxyurea / Thymidine

Hydroxyurea (HU) is an inhibitor of ribonucleotide reductase, which is an enzyme implicated in nucleotide biosynthesis. Thymidine (deoxythymidine) is a pyrimidine deoxynucleoside which pairs with deoxyadenosine in double-stranded DNA and is used for synchronization of the cell in G1/S boundary.

3. Roscovitine

Roscovitine is an olomoucine-related purine flavopiridol that potentially inhibits kinase activity of cyclin-dependent kinase 1 (Cdk1), Cdk2, Cdk5, Cdk7 and Cdk9 (IC_{50} ~ 0.5-0.2mM), which are key regulators of the cell cycle and transcription. Roscovitine is a poor inhibitor for Cdk4 and Cdk6 (IC_{50} > 100mM).
Roscovitine inhibits the kinase activity by competing with ATP at ATP binding site of CDK.

4. Caffeine / UCN-01

Caffeine is a methylxanthine and used widely for inhibiting ATM-ATR kinase activity in DNA damage response. UCN-01 (7-hydroxystaurosporine) is known as a potent Chk1 inhibitor.

5. UO126

UO126 is specific inhibitor of mitogen-activated protein kinase kinase (MEK 1/2).

F. Cell Cycel Analysis (Flowcytometry)

Cells were trypsinized with 1X EDTA-trypsin and collected by the centrifugaton at 1,000 rpm for 5 min with PBS washing. The pellet was fixed with 90% methanol at -20°C more than 2hrs. Methanol fixed cells were centrifuged at 1,000 rpm for 5 min. The supernatant was removed and pellet was resupended with PBS for washing by brief vortexing. Cells were re-collected by the centrifugation at 1,000 rpm for 5 min and PBS was removed. Cells were incubated with primary antibody MPM2 (1:500) in PTB buffer (3% BSA in PBS, 0.02% Triton-X) at 37°C for 1hr. After centrifugation at 1,000 rpm for 5 min, supernatant was removed and cells were washed with PBS. Samples were incubated with anti-mouse FITC-conjugated secondary antibody (1:250) for 1hr at 37°C. Followed by brief PBS wash with centrifugation, DNA was stained with Propodium Iodine (PI) in Sodium Citrate including RNase A buffer (10μg/ml Propidium Iodine, 4mM Sodium Citrate/PBS, 0.1% Triton X-100, 0.1M Tris, 30U/ml RNase A, 0.1M NaCl, 5mM EDTA) for 10 min at 37°C. Staining reaction was stopped by direct incubation on the ice.

Samples were analysed with FACS machine using CellQuest software from Becton and Dickson Company.

G. siRNA transfections

HCT116 cells were plated at a density of $1X\sim2X10^5$ cells/$6cm^2$ dish. Cells were transfected with RNA oligomers using Oligofectamine (Invitrogen) for 4~6hrs in serum-free medium, then 20% of serum was added.
The siRNAs were synthesized by Dharmacon (Lafayette, CO), and the sequece of oligonucleotides used for control was :

Ctrl 5'-CUUACGCUGAGUACUUCGAdTdT-3'

The siRNA oligomers used for Emi1 silencing were 21 bp synthetic molecules with :

5'-AAACUUGCUGCCAGUUCUUCAUU-3' (A)
or 5'-AAGCACUAGAGACCAGUAGACUU-3' (B)

The sequences of RNAi used in experiment were below as followed :

β-TrCP1/2	5'-GUGGAAUUUGUGGAACAUCdTdT-3'
Chk1	5'-UCGUGAGCGUUUGUUGAACdTdT-3'
Cdc20	5'-CGGCAGGACUCCGGGCCGAdTdT-3'
Cdc34	5'-GCUCAGACCUCUUCUACGAdTdT-3'
Cdh1	5'-UGAGAAGUCUCCCAGUCAGdTdT-3'
Cul1	5'-UAGACAUUGGGUUCGCCGUdTdT-3'
Cul4A	5'-GAAGCUGGUCAUCAAGAACdTdT-3'
Cul4B	5'-AAGCCUAAAUUACCAGAAAdTdT-3'
Evi5	5'-CCUCAGUCACCUUGAAGAAUU-3'
p21-H1	5'-CUUCGACUUUGUCACCGAGdTdT-3'
Plk1	5'-GGGCGGCUUUGCCAAGUGCdTdT-3'
Rb	5'-GUUGAUAAUGCUAUGUCAAdTdT-3'

H. Immunofluorescence

Cells were plated on glass coverslips coated with poly-L-lysine, rinsed in PBS, and fixed with 4% paraformaldehyde/PBS for 20 min in 37°C incubator or with cold absolute methanol in freezer (-20°C), respectively. Fixed cells were permeabilized with PBS/0.2% Triton X-100 for 3 min, washed in PBS for 3 times, and primary antibody was added 3% BSA/PBS/0.5% Tween 20 buffer for 1hr incubation at 37°C. After 3 time washes with PBS, the coverslips were incubated with FITC-conjugated secondary

antibody for 1 hr at 37°C. Cells were rinsed with PBS for 3 times and DNA was stained with Propodium Iodine in RNase A/PBS for 10 min at 37°C in dark. After the incubation was finished, cells were washed 3 times with PBS. Then samples were mounted by using DABCO solution.

I. Specific Immunodetection of BrdU

The utilisaition of BrdU (bromodeoxyuridine, Sigma), nucleotide analog, permits identification of the cell passage in the course of S phase. BrdU incorporates with DNA during replication. Cells were grown on poly D-lysine coated coverslips and incubated in the presence of BrdU for 30 min at 37°C and then fixed with 70% ethanol. After washing with PBS, fixed cells were incubated with 2N HCl / 0.5% Triton-X for 10 min and then immediately neutralized with 0.1M Sodium tetraborate for 5 min. Cells with denatured DNA were washed once with PBS and anti-BrdU antibody conjugated with FITC (1:30) was added in 3% BSA/PBS/0.5% Tween 20 buffer for 1 hr at RT. Cells were washed with PBS and DNA was stained with 5μg/ml Propidium iodine in PBS for 5 min at 37°C.

J. Bacterial transformation, Plasmid preparation and DNA transfection

Escherichia Coli (*E. Coli*) DH5α competent bacteria were used for plasmid preparation. 50-100μl of competent cells were put in a 1.5ml Eppendorf tube on ice. 500ng of desired plasmids containing ampicillin resistant gene were added into *E. Coli* cells and incubate on ice for 10 min. Mixed tubes were incubated in water bath at 42°C for 2 min and rapidly the tube was transferred to ice for 2 min to reduce damage to *E. Coli* cells. 2ml of LB (Trypton, Yeast extract, NaCl, pH7.2) without antibiotic was added and then tubes were incubated for 1.5 hr at 37°C. 100μl of transformed cells were spread on LB agar plate with ampicillin and grown in incubator at 37°C for overnight. After colonies formed in plate, transformed bacteria colonies were picked and cultured in 200ml of LB media with ampicillin in shaking incubator at 37°C for overnight. Maxi plasmid purification was followed by manufacturing manual (Qiagen).

HCT116 cells were cultured in $10cm^2$ dish with 60-70% confluency. 10μg of desired plasmid and 1μg of purobabe plasmid were mixed with 12μl of Transfast^{TM} transfection reagent (Promega) in serum free media and incubated for 10-15min at RT. The growth media from the cells were removed and cells were briefly washed with PBS. The mixture was added and incubated for 1hr at 37°C and then 20% of serum was added.

K. Immunoprecipitations (IP)

Protein extracts (300μg) were pre-cleared with 20μl of Protein A(or G)-Sepharose (Sigma) in 0.5ml effendorf tube for 2hr or overnight at 4°C and desired antibodies were also incubated with PA(G)S beads for 1hr at 4°C. After brief washing with IP buffer (Tris 50mM, NP-40 0.5%, NaCl 150mM, Glycerol 10%) and centrifugation at 6,000 rpm for 10 sec, pre-cleared protein supernatant was added to antibody conjugated beads and incubated for 2hr at 4°C. After incubation, the beads were washed 5 times with ice-cold IP buffer. Proteins were recovered by heating at 100°C for 3 min in 4x sample buffer (0.2M Tris, 2% SDS, 20% β-mercaptoethanol, 40% glycerol, 0.1% bromophenolblue).

L. Histone H-1 Kinase Assay

20μl of Protein A(or G)-Sepharose in 40mM HEPES buffer pH7.5 (ratio 1 :1) was added into 0.5ml effendorf tube and washed with IP buffer (Tris 50mM, NP-40 0.5%, NaCl 150mM, Glycerol 10%) briefly. After centrifugation at 6,000rpm for 10 sec, the supernatant was removed and 1-3μl of primary antibody was added and incubated with 10μl of IP buffer with Sepharose beads for 1hr at 4°C. Beads were washed with IP buffer and centrifuged at 6,000rpm for 10 sec. The supernatant was removed and 100~300μg of protein extract was added. The mixture was incubated for 1-2hr at 4°C. Immunoprecipitated beads were spun at 6,000rpm for 10 sec and washed with IP buffer for 3-5 times and then washed twice with kinase buffer (40mM HEPES pH7.5, 8mM $MgCl_2$). All buffer was removed and beads were incubated with 18μl of reaction solution (2μl of Histone H-1 (2mg/ml), 1μl of ATP (3mM), 0.5-1μl of γATP, 9μl of 2x kinase buffer per reaction) at 37°C for 20 min. The reaction was stopped by adding 6μl of 4x sample buffer and the mixture was heated at 100°C for 3 min, and then spun briefly before loading to the gel. 10μl of each sample was loaded to 12% acrylamide gel. The gel was stained with coumassie blue staining solution (2.5g/l of coumassie brilliant blue, 50% Methanol, 13% Glacial acetic acid) for 30 min and then destained with destaining solution (10% Methanol, 10% Glacial acetic acid) for 1 hr-overnight. Destained gel was rinsed with water for 1hr and dried in vacuum condition at 80°C for 2 hr. The film was exposed usually for 1-2 hr at -80°C or RT.

M. Plk (Polo-like kinase) Activity Assay

1μl of Plk primary antibody was incubated with 10μl of Protein A-Sepharose beads at 4°C for 1 hr in lysis buffer (150mM NaCl, 50mM Tris, 0.5% NP-40, 10mM NaF, 1mM PMSF, leupeptin, aprotinin). Protein extract (300-500μg) was thawed on ice and added immediately to the beads for incubation at 4°C for 1-2 hr. The mixture was washed with 4x lysis buffer containing 150mM NaCl and then, washed once with kinase buffer (10mM HEPES, 150mM KCl, 10mM $MgCl_2$, 2mM DTT, 1mM EGTA). All buffer was removed and 16μl of the reaction solution (1μg of Casein, 1μl of 3mM ATP, 0.5μl of γATP, 8μl of 2x kinase buffer / reaction) was added. The mixture was incubated at 37°C

for 20 min. The reaction was stopped by adding 4x sample buffer and the mixture was heated at 100°C for 3 min for loading to 12% acrylamide gel. After separation, the proteins was transferred to the nitrocellulose membrane for 1 hr, and then the film was exposed for 2-4hr at RT.

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